EVALUATION OF SMART POLYMERS FOR CONTROLLED RELEASE DELIVERY SYSTEMS

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Our goal was to develop a smart polymer, controlled release delivery system and evaluate its capabilities for use with salmon calcitonin and rivastigmine. Thermosensitive and phase sensitive smart polymers were evaluated for their potential as controlled release delivery systems. Thermosensitive triblock copolymers were synthesized with increasing lactide to glycolide ratios of 3.5:1, 4.5:1, and 5:1. Characterization was via analytical techniques including proton nuclear magnetic resonance, gel permeation chromatography, critical micellar concentration, sol-gel transition test tube inversion, and cellular biocompatibility assay. Only the 5:1 lactide to glycolide copolymer transitioned into gel at body temperature. Release duration *in vitro* was 70 days when salmon calcitonin was incorporated at 40% (w/v) in 5:1 thermosensitive copolymer while retaining the native conformation of salmon calcitonin as analyzed via micro bicinchoninic acid assay, circular dichroism and differential scanning calorimetry. Optimization of thermosensitive and phase sensitive copolymers for delivery of rivastigmine was extensively studied thereafter by comparing key variables of: rivastigmine hydrophobicity, polymer concentration, rivastigmine concentration, and depot volume. The optimal thermosensitive formulation was composed of 35% (w/v) copolymer at an injection volume of 0.5 ml containing 40 mg/ml of rivastigmine base. The release of rivastigmine base was observed for ~16 days in a zero-order fashion. For phase sensitive polymer, we found the best formulation after optimization was that of 5% (w/v) 50:50 poly(lactic-co-glycolic acid) in 95:5 benzyl benzoate to benzyl alcohol with rivastigmine base incorporated at 216 mg/ml. Release was observed over the course of ~42 days. *In vivo* testing was performed using the optimized phase sensitive smart polymer composed of 50:50 PLGA at 5% (w/v) in 95:5 benzyl benzoate with rivastigmine tartrate incorporated as a suspension. Using this formulation, we achieved controlled release for

7 days. Acetylcholinesterase activity was evaluated in the brains of the rats at different time points for all conditions. Acetylcholinesterase was inhibited during controlled release of rivastigmine by 42% in 7 days, compared to healthy controls. The results demonstrate that controlled release of rivastigmine was accomplished and shows promise as a method to increase dosing interval and improve quality of life for those suffering from Alzheimer's Disease.

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DEDICATION

To all of my family for without your encouragement, support, and motivation none of this would have been possible (especially to Easton Mathias Lipp and Ruth Jean Lipp; you kept me going when it felt like I couldn't or didn't want to). Also dedicated to all women in STEM, because we all know it hasn't been easy to get where we have, and it will continue to be a challenge every day. Persist.

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LIST OF ABBREVIATIONS

LA	Lactic Acid/ Lactide.
PLA	Poly Lactic Acid/ Polylactide.
GA	Glycolic Acid/ Glycolide.
PGA	Poly Glycolic Acid/ Polyglycolide.
PLGA	Poly Lactic Co-Glycolic Acid.
mPEG	Monomethoxy Polyethylene Glycol.
mPEG-PLGA-mPEG	Triblock Copolymer of Monomethoxy Polyethylene Glycol and Poly Lactic Co-Glycolic Acid.
sCT	Salmon Calcitonin.
^{1}H NMR	Proton Nuclear Magnetic Resonance.
GPC	Gel Permeation Chromatography.
CMC	Critical Micelle Concentration.
HEK293	Human Embryonic Kidney Cell Line.
Mn	Number Average Molecular Weight.
Mw/MW	Weight Average Molecular Weight.
PDI	Polydispersity Index.
PBS	Phosphate Buffer Saline.
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl-Tetrazolium Bromide.
DMSO	Dimethyl Sulfoxide.
DMEM	Dulbecco's modified Eagle's medium.
BB	Benzyl Benzoate.
BA	Benzyl Alcohol.

RT .....Rivastigmine Tartrate.

CD .....Circular Dichroism.

DSC .....Differential Scanning Calorimetry.

PEO .....Poly (Ethylene Oxide).

PPO .....Poly (Propylene Oxide).

IPDI .....Isophorone Diisocyanate.

PLLA .....Poly L-Lactic Acid.

FTIR .....Fourier-transform Infrared Spectroscopy.

CDCL3 .....Deuterated Chloroform.

TMS .....Tetramethylsilane.

LCST .....Lower Critical Solution Temperature.

UCST .....Upper Critical Solution Temperature.

WAXD .....Wide Angle X-ray Diffraction.

NIPAAM .....N-Isopropylacrylamide.

C_{max} .....Maximum Serum Concentration.

AUC .....Area Under the Curve.

NMP .....N-Methyl-2-Pyrrolidone.

SAM .....N-Stearoyl L-Alanine Methyl Ester.

t_{max} .....Time to Reach C_{max}.

PTH .....Parathyroid Hormone.

AD .....Alzheimer's Disease.

NMDA .....N-Methyl D-Aspartate.

Aβ .....Amyloid Beta.

ADMET	Absorption, Distribution, Metabolism, Excretion, Toxicity.
BuChE	Butyryl Cholinesterase.
AChE	Acetyl Cholinesterase.
PBCA	Poly (N-Butylcyanoacrylate).
CSF	Cerebral Spinal Fluid.
IL	Interleukin.
HRT	Hormone Replacement Therapy.
LA:GA	Ratio of Lactide to Glycolide.
RP-HPLC	Reverse Phase High Performance Liquid Chromatography.
w/v…	Weight to Volume Ratio.
BSA	Bovine Serum Albumin.
BB:BA	Ratio of Benzyl Benzoate to Benzyl Alcohol.
kDa	Kilodalton.
HED	Human Equivalent Dose.
AED	Animal Equivalent Dose.
DNTB	Ellman's Reagent (5,5'-dithiobis-(2-nitrobenzoic acid).

1. INTRODUCTION

1.1. Copolymers of Polylactide and Polyglycolide

Resorbable comes from the word resorb which means to be absorbed again. In context of drug delivery, resorbable means to be broken down and assimilated in the body. Diblock and triblock copolymers composed of PLA and PGA are bioresorbable which means the broken-down parts of these polymers will get absorbed or dissolved in the body.[1,2] Block copolymers are a specific type of polymer which constitute different blocks or sections of polymerized monomers. A diblock copolymer is composed of two different chemical blocks, such as PLA-PLGA and a triblock copolymer is composed of three different chemical blocks where each block has at least one feature absent in the adjacent sections, such as PLA-PLGA-PLA. The basic units (monomers) of PLA and PLGA are lactic acid and glycolic acid. PLA is generally synthesized by the ring opening polymerization of two monomers lactic acid and the cyclic di-ester lactide using a metal catalyst (e.g. stannous octoate). The polymer PLA exists in an optically active form (L-PLA) which is semi-crystalline in nature, and an optically inactive racemic form (D,L-PLA) which is an amorphous polymer because of irregularities in its polymer chain structure. D,L-PLA forms a more homogenous dispersion of drug in polymer matrix and therefore is the preferred choice over L-PLA for controlled drug delivery systems.[1,2,6,7] Polyglycolide or poly(glycolic acid) (PGA) is a polymer formed by the polycondensation of glycolic acid or most commonly by the ring-opening polymerization of cyclic diester of glycolic acid, glycolide.[6,7] PGA is hydrolytically unstable and degrades rapidly by random hydrolysis and cellular enzymatic activity, owing to the ester linkage in the backbone, to form glycolic acid which is consumed by the cells via the citric acid cycle. Expeditious degradation leading to low mechanical strength usually limits its application as a biomaterial. PLA as compared to glycolic

acid is more hydrophobic due to the presence of an extra methyl group resulting in resistance to hydrolysis and degradation. Consequently, to optimize the degradation rate and pattern, PLA is often copolymerized with other degradable polymers such as PGA and polyethylene glycol (PEG) which are comparatively hydrophilic in nature.[1] Additionally PEG serves as a protective layer against the immune system.[2]

Biodegradable copolymers of ABA and BAB triblock were introduced by MacroMed, where A represents the hydrophobic polyester block (PLA or PLGA), and B represents the hydrophilic (PEG) block. Some examples of biodegradable triblock copolymers include PLGA-PEG-PLGA, PLA-PEG-PLA, and mPEG-PLGA-mPEG (Figure 1). Due to these hydrophobic and hydrophilic moieties, these polymers have the ability to form temperature sensitive polymeric micelles. These polymeric micelles resemble natural carriers (such as viruses and serum lipoproteins) owing to a hydrophilic shell allowing them to circulate in the blood stream for a longer period unharmed by the immune system with the small size preventing from uptake by the reticuloendothelial system (RES), and the hydrophobic core enabling protective encapsulation of drugs/proteins/peptides.[1] The main objective of this chapter is to summarize the history, synthesis, and characterization of PLA and PGA based diblock and triblock copolymers along with the application of these copolymers as resorbable drug delivery systems.

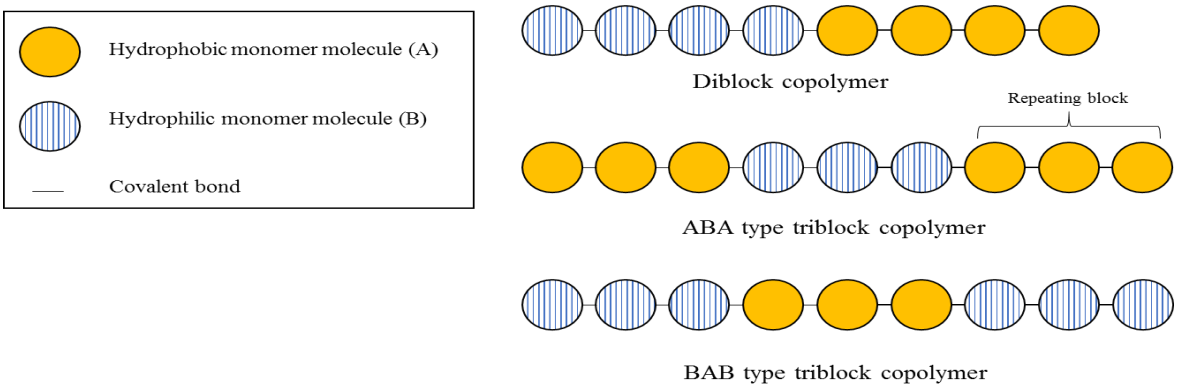

Figure 1. Schematic Representation of Diblock and Triblock Copolymers of Different Types.

1.1.1. History of PLA

PLA is synthesized using two main monomers lactic acid, and the cyclic di-ester, lactide. It has a melting temperature from 180-220 °C and a glass transition temperature of 60-65°C.[1] It has a density of 1.210–1.430 g·cm−3 and is insoluble in water. Lactic acid was discovered in 1780 by a Swedish chemist, Carl Wilhelm Scheele, who isolated it as an impure brown syrup from sour milk.[3] By early 1880s lactic acid was commercially produced in the US marking the first step towards the study of lactic acid polymers. In 1845, PLA was first synthesized by the condensation of lactic acid,[4] and later using reversible polymerization of cyclic esters by heating lactic acid under vacuum.[5] Soon after that PLA had started being used commercially as a fiber material for resorbable sutures.[6] PLA produced by these methods was expensive and of low molecular weight. Breakthrough research by Cargill Inc. in the early 1990s made acquainted the production of high molecular weight PLA using a commercially viable lactide ring opening polymerization reaction.[7] The direct condensation route was an equilibrium reaction which made it difficult to remove traces of water at high conversion stages in order to drive the reaction to higher molecular weight. Additionally, the polymerization method used by Cargill Inc. involved synthesizing both lactide and PLA in the melt thereby avoiding the use of costly and unfriendly solvents. Firstly, a low molecular weight PLA prepolymer was produced by continuous condensation reaction of aqueous lactic acid, following which high molecular weight PLA was synthesized using a tin-catalyzed ring-opening lactide polymerization reaction. Unreacted lactide was recycled to the beginning of the process by vacuum distillation. The major advantage of this process was the selectivity of the intramolecular cyclization reaction to add from a mixture of lactide stereoisomers using tin catalysis involving coordination-insertion mechanism with more than 90% conversion and extremely low rate of racemization.[7]

1.1.2. History of PGA

PGA was one of the very first resorbable polymer investigated to be used as a biomaterial. It is synthesized from glycolic acid which is a colorless, odorless, hygroscopic, and crystalline solid with high water solubility. PGA has been known since 1954 as a biodegradable, tough fiber-forming polymer largely used for forming synthetic absorbable sutures (Dexon) of high strength and modulus, as well as medical implants.[8] PGA is a highly crystalline polymer (45-55%) with a glass transition temperature ~35 ºC and a high melting point in the range 225-230 ºC. It is insoluble in water and most organic solvents. Fluorinated solvents such as hexafluoroisopropanol and hexafluoroacetone sesquihydrate are unique in the capability of dissolving PGA allowing its spinning or molding into cast films.[9] Several methods have been investigated for the synthesis of PGA. Polycondensation of glycolic acid is the simplest way of synthesizing PGA by heating glycolic acid at 175-185 ºC to distill off water followed by continued heating at reduced pressure for few hours to obtain a low molecular weight byproduct glycolide. A ring opening polymerization method to synthesize PGA was invented by heating pure glycolide under nitrogen atmosphere in the presence of antimony, zinc or tin containing compounds as catalysts. At present, stannous octoate is the most commonly used catalyst for this reaction. The reaction is carried out at a temperature below the melting point of PGA and the reactants allowed to react for about 30 minutes to obtain high molecular weight PGA.[10] In another study, solid state polycondensation of halogenoacetates (e.g. sodium chloroacetate) under nitrogen atmosphere in round bottom flask have also been used effectively to synthesize high molecular weight PGA.[11]

1.2. Synthesis of Diblock and Triblock Copolymers of PLA and PGA

Ring opening polymerization is the most widely used method for the synthesis of diblock and triblock copolymers of PLA, PLGA and PEG where the hydrophobic A block is covalently linked to the hydrophilic B block by an ester linkage.[12] Various authors have used ring opening polymerization method to synthesize different polymers of varying copolymer compositions. The scheme of synthesis remains analogous. For example, diblock copolymer (PEO:D,L-PLA) synthesis can be achieved by taking equal molar ratios of PEO (poly(ethylene oxide)) and D,L-lactide in a round bottom flask. Solvent toluene and nitrogen atmosphere are used to obtain an anhydrous atmosphere for the reaction and stannous octoate is used as a catalyst. The reaction is carried out under reflux to prepare the diblock copolymer. The diblock copolymers can be coupled to synthesize triblock copolymer (PEO-PLA-PEO) using isophosphodiisocyanate (IPDI) (a coupling agent) dissolved in toluene and refluxed with the diblock copolymer. The obtained copolymer is purified using fractional precipitation from methylene chloride using diethyl ether.[13] Similarly, mPEG-PLGA diblocks can be synthesized and coupled using IPDI to prepare mPEG-PLGA-mPEG triblock copolymer.[14]

On the other hand, synthesis of PLA-PEG-PLA triblock copolymer is a one-step process with no intermediate step for coupling. Briefly, calculated molar ratios of PEG (initiator) and D, L-lactide are taken, and D,L-lactide is charged into a three-necked flask containing pre-dried PEG in anhydrous toluene under nitrogen atmosphere. Once all the reactants are in molten state, stannous octoate is added as the catalyst and the reaction is carried out at 120 ºC for 12 h to synthesize the triblock copolymer of desired copolymer composition. The copolymer obtained by this method can be purified by dissolving the crude copolymer in ice cold water followed by

precipitation by heating. This purification step is repeated 2-3 times to remove unreacted monomers and impurities. The final product is freeze dried to remove the residual water.[15,16]

Using the above-mentioned ring opening polymerization method, copolymer of different block lengths can be achieved by varying the feed ratio of the monomers and the initiator. Studies have suggested that a larger hydrophobic block leads to sustained degradation of the resorbable polymer matrix resulting in controlled delivery of the incorporated drug over a long period. The ratio of hydrophilic and hydrophobic block lengths also affects the aqueous solubility and sol-gel transition temperature of the respective copolymer. In different articles Singh and coworkers varied the block lengths of both the hydrophobic and hydrophilic blocks while conserving the copolymer's water solubility, injectability at room temperature, sol-gel transition ability, and stability of the gel at 37 ºC.[14,15,17–21]

A different method was employed by Wu et al. to synthesize MPEG-b-PLA diblock copolymer using monomers MPEG and LA and carrying out the copolymerization reaction in oil bath at 140 ºC for 48 h. The precipitate obtained was cooled to room temperature and purified by dissolving in anhydrous methylene chloride followed by precipitating out the copolymer using ethyl ether. MPEG-b-PLA obtained was then dried under vacuum and was used as a macroinitiator, owing to the hydroxyl groups present on its backbone which can initiate ring opening polymerization of cyclic poly (ethyl ethylene phosphate) (EEP), to generate methoxypolyethylene glycol-poly (D, L-lactide)-poly(ethyl ethylene phosphate) (MPEG-b-PLA-b-PEEP) triblock copolymers, in an additional synthesis reaction.[22]

Transesterification reaction of PLA with PEGNH2 was also explored by a group of authors to synthesize PLA-b-PEG copolymer.[23] Powder form of PLGA-PEG-PLGA triblock copolymers can be synthesized by first preparing copolymer PLGA in powder form using direct

melt polycondensation method. Then to synthesize PLGA-PEG-PLGA triblock copolymer in powder form, different proportions of PLGA can be added to a fixed amount of PEG with stannous octoate as the catalyst and the mixture heated in a two necked round bottom flask under nitrogen atmosphere to obtain a crude brown colored product.[24] Another study demonstrates the synthesis of PLA/PEG triblock and multiblock copolymers using acyl halide-terminated PLA (PLA-diCOCl) prepolymer and anhydrous pyridine.[25]

Recently, thermosensitive star shaped block copolymers have been investigated for their application as injectable copolymeric drug delivery system.[27] The copolymers constituted of fixed molecular weights of PEG, and varied mole ratios of D,L-lactide to glycolide (PLGA block) and overall feed ratios of D,L-lactide, glycolide, and 3 or 4 arm PEG. The monomers are added into a round-bottom flask under nitrogen atmosphere where the 3 or 4 arm PEG act as a multifunctional initiator and stannous chloride acts as a catalyst. Bulk ring opening polymerization is performed and the copolymer product obtained is cooled to room temperature and precipitated for purification using diethyl ether several times.[26] A three-step synthesis mechanism was also recently invented to synthesize 3-arm star-shaped PLGA-mPEG (3sPLGA-mPEG) and 4-arm star-shaped PLGA-mPEG (4sPLGA-mPEG) copolymers using arm-first method.[27] In this method, linear chain hydroxyl-terminated PLGA-mPEG diblock copolymer (LPLGA-mPEG) is synthesized by bulk ring opening polymerization followed by carboxylation of dried trimethylolpropane (TMP) or pentaerythritol (PTOL) to produce CTMP or CPTOL with three or four carboxyl acid terminal groups using excess amount of succinic anhydride (SA). Finally, esterification reaction of two reactive precursors, L-PLGA-mPEG and CTMP or CPTOL, is performed using 1,3-dicyclohexylcarbodiimide (DCC) as a dehydrating agent and 4-

(dimethylamino) pyridine (DMAP) as a catalyst to obtain 3-arm or 4-arm star-shaped PLGA-mPEG block copolymer.

1.3. Characterization of Copolymers of PLA and PGA

1.3.1. Structural Composition Analysis

The confirmation of completion of polymerization reaction of PLA-PEG-PLA, and PLGA-PEG-PLGA triblock copolymers along with the molecular structure can be determined using Fourier transform infrared spectrometer in the frequency range 4000–1000 cm−1 in absorbance mode. The characteristic peak of the carboxylic acid of PLA from 1700-1725 cm−1 disappears and a new peak appears in the region of 1730–1750 cm−1 due to the newly formed ester groups in the FTIR spectra. The characteristic peak for isophoronediisocyanate, used as a coupling agent, at 2175 cm−1 can be used as an indicative of completion of coupling reaction in PEO:D,L-PLA:IPDI:D,L-PLA:PEO type coupled diblock copolymers 13. Characteristic signals of PEG ether band and PLA ester carbonyl band can also be seen at 1086 cm-1 and 1755 cm-1, respectively, in a such a diblock or triblock copolymer.[23]

Additionally, both proton (1H) and carbon (13C) nuclear magnetic resonance (NMR) are established techniques to determine the chemical structure and structural composition of PLA and PGA block copolymers. The analysis may be performed using NMR spectrometer operating at 300 or 400 MHz. The copolymer is dissolved in an organic solvent such as deuterated dimethyl sulfoxide (DMSO-d6) or deuterated chloroform (CDCl3) with tetramethylsilane (TMS) signal as the internal reference standard. Resonances in ~5.2-5.0 ppm range (-O-CH) and ~1.5-1.4 ppm (CH3) belong to PLA blocks. The main chain methylene signals of PEG (in a PLA-PEG-PLA triblock copolymer) usually show at 3.7-3.3 ppm range. The α-methylene protons (PLA-COO-CH2) and hydroxylated methine (-CH) protons of lactyl end units appear together in

the range ~4.3-4.1 ppm. If the copolymerization did not take place effectively carboxylated end units of lactyl and methine protons of free lactic acid appear at ~5.0-4.0 ppm range and 4.03 ppm, respectively, in a 1H NMR spectrum.[28] Similar characteristic peaks were reported by several other authors with slight variations complying with the change in the type of block copolymer, and the monomers in the copolymer chain backbone.[14,15,29] The structural composition, graft ratio, and the number average molecular weight (Mn) of the polymers can then be calculated by analyzing the integrated signals corresponding to chemical groups -CH and -CH3 of LA, and -CH2 of EG in 1H NMR.[16,23] The spectrum of 13C NMR has also been used to confirm the presence of PEG and PLA blocks by the characteristic peak of methylene (-CH2) group of PEG block at ~ 71 ppm, and carbonyl (-C=O), methine (-CH) and methyl (-CH3) groups of PLA block at ~170, ~69.4 and ~17 ppm, respectively.[16]

Gel Permeation Chromatography (GPC) is used to further determine the number average molecular weight (Mn), weight average molecular weight (Mw), and the molecular weight distribution (polydispersity index, PDI) of the synthesized copolymer. Polystyrene standards are used for calibration and tetrahydrofuran is a popularly used carrier solvent for the GPC analysis of PLA/PEG copolymers.[16,25]

1.3.2. Aqueous Solubility and Injectability

Aqueous solubility and injectability are two very lucrative properties making the copolymers of PLA/PGA versatile for drug delivery use, the main advantage being avoidance of toxic organic solvents. The concentration at which the copolymers are soluble below the gelation temperature is called the functional concentration.[30] The copolymer dissolves in cold water due to the PEG blocks keeping the copolymer in solution and the hydrophobic PLGA/PLA segments forming associative crosslinks. This happens owing to the hydrogen bonding between

hydrophilic PEG blocks and water making the copolymer soluble. This effect is dominant at lower temperatures. The copolymer concentration in water can be varied to allow injectability at room temperature and also to tailor the drug release profile. As the temperature increases the hydrogen bonding gets weaker and the hydrophobic forces in the hydrophobic PLGA/PLA blocks get strengthened and become dominant. This way change in temperature leads to the reversible sol to gel transition of an aqueous copolymer solution of copolymers of PLA/PGA/PLGA.[12] The transition temperature can be varied by changing the hydrophobic and hydrophilic blocks lengths of these copolymers.

For injectable drug delivery application, it is desired that the copolymer solution incorporating the desired therapeutic should be injectable (sol form) at room temperature and on administration at physiological temperature (37 ºC) transform into a stable gel depot at the injection site.[14] The total molecular weight of PLA and PGA copolymers for optimum solubility and reversible thermo-gelation should lie between 3500 to 4100 Da for ABA type and 4000 to 4600 Da for BAB type copolymers. For both types, the average molecular weight of hydrophilic block B (preferably PEG) should fall between 600 and 2200, with the overall weight percentage of the hydrophobic block relative to the hydrophilic block should be preferably high, between 65 and 78%. In BAB type copolymers it has been found that the copolymer composition (ratio of PLA/PEG) and the total molecular weight of copolymer have striking effect on release profile, especially for hydrophilic drugs.[30]

1.3.3. Phase Transition

Response to stimulus is an innate property of living systems. Ability to design a system to manifest this property has been a starting point to several sterling research and inventions. Thermosensitive copolymers of PLA and PGA undergo reversible in situ sol-to-gel transition in

response to temperature changes, figure 2. The transition mechanism of such aqueous copolymeric solutions is related to the presence of both hydrophilic and hydrophobic parts in their structure and usually a lower critical solution temperature (LCST) in aqueous solution.[21] As the temperature increases above LCST the equilibrium shifts from unimers to spherical micelles. The copolymer-water interactions become thermodynamically unfavorable in comparison to water-water or copolymer-copolymer interactions, leading to dehydration of solvated copolymer chains and finally transition into gel state (micelle packing). On increasing the temperature above the upper critical transition temperature (UCST), the polymer precipitates.[16] The transition from gel to sol is related to the shrinkage of the hydrophilic component's corona in the micelles owing to the effect of temperature on its solubility and interaction of its chains with the hydrophobic hard core.[31]

In copolymers containing PEG, the PEG chains orient themselves to align forming the outer hydrophilic shell of the micelles facing the external aqueous environment. This layer of PEG acts as a barrier by reducing interactions with foreign molecules resulting from steric and hydrated repulsion. This results in increased stability and shelf life of such systems.[12,32] It has also been noted that PEG-PLGA-PEG triblock copolymers in aqueous solutions show increased polymer-polymer interaction as compared to polymer-solvent interactions. In other words it has also been suggested that with increase in temperature the polymeric micelles grow by increasing diameter and eventually aggregate driving the sol-gel transition.[33]

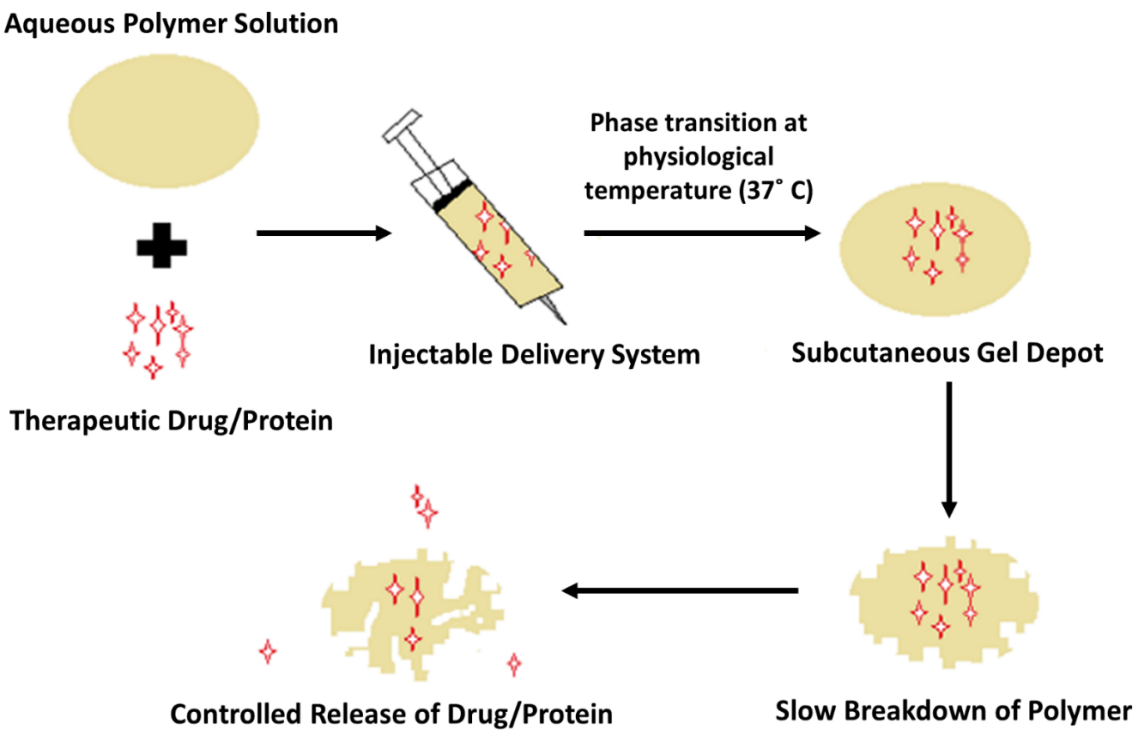

Figure 2. Formulation Schematic and Controlled Release.

1.3.4. Thermal Properties

Thermo gravimetric and differential thermal analysis (TG-DTA) of copolymers provides useful data to assess influence of copolymer composition on the degradation behavior. TGA analyzer instrument is used for the thermal characterization. Gajendiran and group[24] quantitatively assessed PLGA-PEG-PLGA triblock copolymer degradation using thermal characterization method. According to their study PLGA-PEG-PLGA triblock copolymers degrade in two steps with the loss of PLGA at ~300 ºC and PEG at ~410 ºC. Various changes in degradation pattern with the changing copolymer compositions were also reported. In a different study differential scanning calorimeter (DSC) was used to investigate the thermal properties of PLA-PEO-PLA triblock copolymers. It was found that the melting temperature (Tm) of PLA/PEO copolymers was lower than that of PEG alone which ultimately has significant

implications on the glass transition temperature (Tg) and crystallization peak (Tc) of these copolymers.[28]

1.3.5. Crystallization Behavior

The effect of different copolymer compositions and architectures on the thermal properties and crystal structures of block copolymers MPEG-b-PLLA, PLLA-b-PEG-b-PLLA and 4-arm PEG-b-PLLA has been intensely investigated.[34] DSC was used to scrutinize the thermal properties of the copolymers. The instrument was calibrated with pure indium and experiments were performed under nitrogen flow. Heating, cooling and second heating scans were recorded for different MPEG-PLLA block copolymer compositions and the crystallinity of PLLA and PEG was calculated. Alongside, Wide Angle X-ray diffraction (WAXD) measurements were also taken to study the effect of chain connectivity, composition and architecture of these copolymers on the crystal structures of PLLA and PEG. It was reported that the melting point and crystallinity were affected by increasing molecular weight, arm length, and number of arms of PLLA in the MPEG-PLLA and PEG-PLLA block copolymers. This probably happens due to the formation of PLLA crystallites which cause the internment of PEG resulting in increased difficulty for PEG to be packed into the crystal lattice. This suggests that varying architecture and molecular weights of PLA/PEG block copolymers alters the consequential properties and exploring those will open new doors for the application of branched PLA/PEG block copolymers for controlled drug delivery applications.[34] WAXD data was also shown to support the fact that formation of crystalline hydrophobic domains in PLLA gels resulted in higher stiffness while racemic PLA resulted in formation of easily degradable amorphous hydrophobic domains due to the stereo random structure.[35] Thus changing a simple chemical

parameter, i.e. stereo-regularity, can help tailor PLA containing block copolymers such as PLA-PEO-PLA for controlled drug release.

Overall, it has been well investigated and proved that PLA and PEG blocks, in a diblock or triblock copolymer affect the crystallization behavior of each other[36], and the gradually increasing confinement of PEG is dictated by the crystallization of the PLA block.[37] Another study supported this fact by suggesting that crystallizability of PEO blocks depends on its length and can be reduced by copolymerization with PLA blocks.[28]

1.3.6. Biocompatibility, Cytotoxicity, and Biodegradability

In general, a system containing drug suspended in the aqueous solution of PLA/PGA/PLGA based diblock and triblock copolymers causes minimum toxicity and mechanical irritation to the surrounding tissues due to their inherent mucomimetic property, pliability of the gel and biodegradation into lactic acid, glycolic acid and ethylene glycol which naturally dissipate from the body.[30,33] PEG forms the hydrophilic shell of these copolymeric micelles in aqueous medium making them non-immunogenic, biocompatible and soluble in water. It has also been documented that PEG of molecular weight below 30,000 is easily eliminated by the body.[30]

A plethora of data is available on the cytotoxicity and biocompatibility studies of PLA/PGA based copolymers. 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay is a popularly used method to test biocompatibility of a system *in vitro*. The principle of this assay is based on the ability of mitochondrial succinate dehydrogenase present in living cells to reduce this MTT dye to water-insoluble purple formazan crystals. The formazan crystals are then dissolved using isopropanol and the absorbance is measured using a plate reader. MTT assay is useful for assessing subtle toxicity of systems which may not kill cells

rapidly (i.e. within 24-72 hours) but may affect the metabolic and other functions of the cells, necessary to maintain viability. Higher absorbance relates to high viability of the cells and hence low cytotoxicity of the sample tested. The cytotoxicity testing of PLA and PGA constituting block copolymers has been reported with insignificant difference from control (cells incubated with growth medium only) affirming their biocompatibility.[14,15,21,27,38]

In vivo biocompatibility can be tested by injecting animal models with these copolymeric drug delivery systems and comparing injection site skin tissue histology using hematoxylin-eosin (H & E) stain to test for inflammatory responses, masson staining to examine the vascularization, and Gomori's trichome stain to test for collagen deposition, after specific time intervals. The safety and *in vivo* biocompatibility of PLA/PEG based copolymeric systems has been published in various articles. The overall results indicate that up to a week following subcutaneous injection of this copolymeric system, infiltration of neutrophils and macrophages to the injection site occurs demonstrating clear incidence of an acute inflammatory response, which subsides to a milder chronic inflammatory response at 30 days post administration with the presence of a few inflammatory cells, and finally at about 90 days closely resembles the control indicating restoration to normal tissue with no signs of necrosis and/or chronic inflammation.[21,38,39] Biocompatibility of PLA and its copolymers for orthopedic, ophthalmic, otologic, skin, central nervous system, pulmonary system, parotid glands, urinary tract, and cardiovascular applications has also been established with good safety profile compared to conventionally used devices and implants.[40]

The *in vivo* biocompatibility testing of star shaped block copolymers has also been similarly performed.[27] Histology after 30 days showed almost complete restoration to normal tissue and no significant tissue necrosis, hyperemia, edema, hemorrhaging, or muscle damage.

Masson staining experiment showed that vascularization took place after 15 days suggesting that star shaped PLGA-mPEG copolymers supported vascular in-growth, and overall, they have good biocompatibility.

PLA/PEG constituting copolymers degrade by non-enzymatic hydrolysis of ester bonds to non-toxic products which are naturally eliminated by the body processes.[16,25] 1H NMR and GPC can be used to determine the reduction in molecular weight of these copolymers while undergoing hydrolytic degradation. Copolymer composition affects the degree of gel hydration affecting the degradation rate of the copolymer, which in turn affects the permeability coefficient of the incorporated drug through the gel matrix.[39,41] The *in vitro* degradation of block copolymer hydrogels happens similarly by hydrolysis of ester bonds accompanied by the erosion of gel in PBS solution at physiological temperature.[27]

1.4. Resorbable Temperature Sensitive/Thermosensitive Polymers

Temperature sensitive or thermoresponsive polymers are the most widely studied type of stimuli-sensitive smart polymers for drug delivery owing to the ease and benefit of exploiting change in their state in response to physiological temperature. Numerous research papers are fortifying evidence that drug delivery using thermosensitive polymeric systems is progressing at a rapid rate.

Poly(N-isopropylacrylamide) (poly-NIPAAM) was the first most extensively studied prototype of thermosensitive polymers.[14] It was synthesized in the early 1950s by free radical polymerization of N-isopropylacrylamide.[42] However, due to its toxicity as well as low mechanical strength, poly-NIPAAM did not succeed for drug delivery applications.[43] Later, ABA type of nonionic triblock copolymers (Poloxamers), containing poly(ethylene oxide) as the hydrophilic block B and poly(propylene oxide) as the hydrophobic block A (poly(ethylene

oxide)-co-poly(propylene oxide)-co-poly(ethylene oxide) (PEO–PPO–PEO) copolymer, also called Pluronics®) received FDA approval as a pharmaceutical excipient, but could not progress further as a pharmaceutical drug delivery system due to non-biodegradability of the hydrophobic PPO block and related toxicity.[44]

In 1997, MacroMed Inc. replaced the non-biodegradable block in Pluronics® by biodegradable and biocompatible PLA block to develop PEO–PLA–PEO thermosensitive triblock copolymer.[29] In an effort to optimize the phase transition behavior, degradation pattern, and henceforth the drug release kinetics, several copolymer compositions have been investigated over the years with encouraging results.

Broadly, a mixture of the biodegradable/biocompatible copolymer and drugs/protein/peptide can be prepared by simple mixing in aqueous copolymer solution below gelation temperature to form a partially dissolved (colloidal state dispersion such as suspension, or emulsion) or completely dissolved drug delivery system which could be injected parenterally, or administered topically/transdermally, and/or inserted into a cavity (ocular, vaginal, transurethral, rectal, nasal, oral, or aural). On administration, the formulation would undergo thermal gelation at physiological temperature (typically body temperature being above the transition temperature) forming a depot entrapping the drug in the polymer matrix.[45] The release from these copolymeric delivery system follows two mechanisms acting simultaneously: diffusion of incorporated drug, and degradation of polymer matrix. Mostly, the initial release is diffusion-controlled, and the later stage is a combination of both with degradation being dominant.[41] Thermosensitive copolymers of PLA and PGA retain water equivalent to ~10% of the total weight of the hydrogel, which allows for the swelling of the gel depot and a diffusion pathway for the incorporated drug molecules. The water retention has been observed to vary with

the ratio of the hydrophilic/hydrophobic content in the copolymer.[16,20] It has also been reported that during the erosion of the hydrogel matrix (in the later phase) there is a preferential loss of hydrophilic segments (PEG-rich) rendering the remaining gel matrix hydrophobic with reduced water retention and swelling resulting in decreased copolymer degradation ultimately leading to reduced drug release.[12] Additionally, the drug release profile can also be altered by varying the copolymer concentration.[16] Concentrations between 10-30% w/w are most preferred for drug delivery as lower concentrations were found to transition to form a weak gel, and higher concentration are too viscous to be injectable. Optimization is required to reach a balance between a strong gel network and desired release rate.[30] A model hydrophilic drug (ketoprofen) and a model hydrophobic drug (spironolactone) were tested by Jeong and coworkers using PEG-PLGA-PEG thermosensitive copolymer to assess the release model of such a copolymeric system.[41] A domain structure was assumed with the drugs partitioning between hydrophilic shell domain and hydrophobic core domain. Drug release from the hydrophilic shell can be explained by diffusion and that from the hydrophobic core by the modified Higuchi equation.

Thermosensitive copolymers made of PLA and PGA have the advantages of being easy to manufacture, soluble in water, avoidance of toxic organic solvents, simple formulation, ease of administration, controlled release of the incorporated drug, and ability to adjust copolymer composition for controlling the release period by modifying the degradation rate, permeability of the matrix and hence the drug release profile. Drug delivery systems using thermosensitive diblock and triblock copolymers of PLA and PGA will be discussed in detail in the following section.

1.4.1. Thermosensitive Polymer-Based Drug Delivery Systems

The use of amphiphilic block copolymers for drug delivery was first proposed in early 1980s.[46] The innovation of using PLA/PGA/PLGA/PEG copolymers for drug delivery applications lies in the simplicity of using these copolymers to deliver a wide variety of drugs, hormones, as well as sensitive proteins and peptides with efficacy. Sustained delivery of various such therapeutics is highly desired as the conventional drug delivery methods are far from ideal. Frequent subcutaneous, intramuscular, or intravenous injections at short intervals, daily application of patches which adhere poorly and/or cause irritation, poor oral bioavailability, and short half-life after parenteral administration confronts the need for a better controlled delivery system without toxicity.[17] Thermosensitive copolymers of PLA and PGA have shown good results both *in vitro* and *in vivo* for a large number of such therapeutics, with some currently in the clinical testing phase discussed in the next section.

Succinic anhydride terminated diblock copolymer methoxypoly (ethylene glycol)-b-poly(lactide) (mPEG-PLA-SA) has been investigated to synthesize 7-Ethyl-10-hydroxy camptothecin (SN38) drug conjugated polymeric micelles.[47] SN38, an active metabolite of irinotecan is a potent topoisomerase I inhibitor. Its clinical applicability as an antineoplastic drug is limited by its hydrophobicity and instability of the lactone ring in its structure at physiological pH. Drug conjugates with amphiphilic diblock copolymers allows for the formation of polymeric micelles as drug carriers. Advantages of this micellar drug delivery system include passive accumulation of polymeric micelles in solid tumors via enhanced penetration and retention effect (EPR), increased therapeutic efficacy, reduced side effects, less frequent drug administrations, and improved patient compliance. The chain lengths of mPEG and PLA were shown to have a large effect on particle size of the drug conjugated micelles as well as antitumor efficacy, both *in*

vitro and *in vivo*. Polymer-drug conjugate micelles were found to be less toxic and more efficacious drug delivery systems for cancer treatment. Enhanced controlled release properties were also depicted by this drug delivery system which were mainly due to the shielding effect of hydrophilic mPEG shell against plasma proteins thereby reducing clearance via mononuclear phagocyte system, while the hydrophobic core (due to PLA) showed the ability to incorporate hydrophobic drugs and allowing their controlled release. Similarly, another study reported the solubilizing efficacy of typical amphiphilic block copolymer by studying poorly water-soluble drugs paclitaxel and cyclosporin A.[30]

In another study, ocular pharmacokinetics of dexamethasone acetate was evaluated in rabbits by the micro-dialysis method using 20% w/w PLGA-PEG-PLGA copolymer solution and compared to regular eye drops. A 7-fold higher maximum serum concentration (Cmax) and 7.89-fold larger area under the curve (AUC) was obtained with the thermosensitive in situ gelling copolymer validating enhanced corneal permeability, prolonged precorneal retention, improved bioavailability, and higher drug efficacy.[48] A group of researchers also suggested that various additives such as sugars, surfactants, salts, amino acids, proteins, and other substances can be readily incorporated in these block copolymers as and when required to modify the release characteristics and/or stability of the drug compound.[30] A long acting formulation of exendin-4 (Exenatide, EXT) incorporated in PLGA-PEG-PLGA triblock copolymer was tested with and without excipients (zinc acetate, PEG, and sucrose) to control the burst release of EXT, both *in vitro* and *in vivo*, with promising results.[49] EXT is an incretin-mimetic polypeptide established to enhance glucose-dependent insulin secretion for the treatment of type II diabetes. Due to the viscous environment of the gel the hydrolytic instability of the polypeptide significantly decreased. The addition of excipients reduced the burst release with zinc acetate showing the

best effect. In other studies, controlled release of insulin has been widely studied using this delivery system to be injected subcutaneously. Regel® (PLGA-PEG-PLGA) polymer was used for the controlled delivery of recombinant human insulin for the basal requirement of insulin for up to 15 days.[50] Meanwhile, Zentner and coauthors studied the release of paclitaxel, porcine growth hormone, glycosylated colony-stimulating factor and recombinant hepatitis B surface antigen using Regel® polymer with positive influence on drug effectiveness and stability.[51] Controlled delivery of levonorgestrel, testosterone, and growth hormone has also been investigated from PLGA-PEG-PLGA thermosensitive copolymer-based delivery systems. Effect of varying block lengths on release profiles was observed and conclusions were drawn for the effect of different drug types on the release profile and duration of drug release.[17,18,21]

Recently, to simultaneously utilize the benefits of PLGA-PEG-PLGA, in terms of ease of formulation, localized administration, biodegradability, low systemic toxicity and sustained drug delivery with combination drug therapy, in an effort to improve anti-cancer efficacy of drugs against osteosarcoma, a localized co-delivery system of PLK1shRNA/PEI-Lys complexes and doxorubicin (DOX) suspended in PLGA-PEG-PLGA thermosensitive hydrogel was developed.[39] The delivery system allowed for sustained co-delivery of the incorporated drugs with no cytotoxicity, biocompatibility, and significant synergistic anti-tumor efficacy. Moreover, localized delivery to the tumor was beneficial in reducing systemic toxicity as observed by ex vivo histological analysis of major organs in Saos-2 xenografts models.

Furthermore, controlled delivery of proteins and peptides is a highly challenging effort owing to low half-life, implicit instability and structural constraints. These are also some of the preeminent reasons that render basal level insulin delivery to type I diabetes patients a daunting task. Multiple frequent injections or round the clock insulin pump are conventionally used

nowadays in order to maintain normoglycemia. Delivery of sensitive proteins and peptides has been extensively studied using PLA/PLGA based triblock thermosensitive copolymers. PLGA-PEG-PLGA thermosensitive triblock copolymers showed a controlled release of different proteins for ~2 weeks.[13,50] This copolymer system demonstrated high burst release of hydrophilic drugs like insulin owing to the higher hydrophilic GA content in the copolymer backbone. PLA being more hydrophobic than PLGA was hypothesized to undergo a slower degradation owing to retarded hydration, swelling and hydrolysis, and was further investigated for the controlled basal delivery of insulin. PLA-PEG-PLA triblock copolymers showed significantly lower burst release with desirable zero-order release profile over a period of 2-3 months.[15] Later, by incorporation of chitosan-zinc-insulin complex in PLA-PEG-PLA copolymer, a controlled basal insulin delivery of ~84 days was obtained *in vitro*.[52] In an additional study, biocompatibility of the delivery system and efficacy of the released insulin was successfully confirmed *in vivo* using streptozotocin-induced diabetic rat model.[38]

Simultaneously, an additional advantage observed with these block copolymers is the protection of the incorporated drugs from chemical degradation which is extremely helpful for sensitive protein and peptide-based drugs. Chen et al. (2005) studied the release profile of model protein lysozyme using PLGA-PEG-PLGA thermosensitive copolymer of varying block lengths and aqueous copolymer concentrations. Controlled delivery of lysozyme was reported in a biologically active form, with significant lowering of burst release with increasing copolymer concentration.[19] Similar studies were done with mPEG-PLGA-mPEG copolymer and the effect of extending the PLGA block resulting in decreased degradation and controlled release of the protein for a longer duration was reported.[14] Controlled delivery of salmon calcitonin, a polypeptide hormone for the prevention and management of osteoporosis, was also investigated

using mPEG-PLGA-mPEG triblock copolymer *in vitro* and in female rat model. Calcitonin suspended in 40% w/v aqueous copolymer solution administered subcutaneously was seen to protect the rat from methylprednisolone acetate induced osteoporosis for up to 40 days.[53] Representative examples of sustained release depot based drug delivery systems of polylactide and polyglycolide diblock and triblock copolymers are summarized in Table 1.

Table 1. Representative Examples of Depot-based Drug Delivery Systems of Polylactide and Polyglycolide Diblock and Triblock Copolymers.

Copolymer	Active Ingredients	Major Effects	References
PLGA–PEG–PLGA	Lysozyme	• Increasing the PLGA block lengths of copolymers decreased initial burst release. • Increasing copolymer concentration reduced the rate of drug release	[19]
mPEG-PLA	7-Ethyl-10-hydroxy camptothecin	• Self-assembling micelles forming mPEG-PLA-SN38 conjugates were synthesized. • Passive accumulation of polymeric micelles in solid tumors via enhanced penetration and retention effect was observed *in vitro* and *in vivo*. • Reduced toxicity and increased anticancer efficacy of the system.	[47]
PLGA-PEG-PLGA	Cyclosporin, paclitaxel	• Improved solubilization of poorly water-soluble drugs. • Increased chemical stability.	[30]
PLGA-PEG-PLGA	Dexamethasone acetate	• Enhanced corneal permeability and prolonged precorneal retention. • Increased Cmax and AUC. • Improved bioavailability, and higher drug efficacy.	[48]
PLGA-PEG-PLGA	Exendin-4	• Increased stability. • Possible addition of excipients reduced burst release.	[49]
PLGA-PEG-PLGA	Recombinant human insulin	• Controlled basal insulin release observed up to 15 days *in vitro* and *in vivo* after single s.c. injection.	[50]
PLGA–PEG–PLGA (Regel®)	Paclitaxel, pGH, G-CSF, insulin, rHbsAg	• Reduced clearance of paclitaxel after direct intratumoral injection with minimal distribution into any organ. • Controlled release of paclitaxel for ~50 days. • Controlled release of equivalent amount of pGH, insulin, and G-CSF after single s.c. administration compared to daily i.v. conventional therapy. • Regel®/rHBsAg increased rHBsAg- specific antibody titers by 6 times compared to commercial vaccine Engerix-B®.	[54]
PLGA–PEG–PLGA	Levonorgestrel, Testosterone, Growth hormone	• Increasing the hydrophobic PLGA block length of copolymers significantly decreased the release rate. • Controlled zero-order *in vitro* release was observed. • Enhanced absolute bioavailability of pGH compared to s.c. aqueous pGH solution.	[17,18,21]
PLGA-PEG-PLGA	PLK1shRNA/PEI-Lys complexes and doxorubicin	• Synergistic anti-tumor efficacy of co-incorporated drugs. • Reduced systemic toxicity owing to localized tumor delivery.	[39]
PLA-PEG-PLA	Insulin, Zinc-Insulin hexamers, Chitosan-Zinc-Insulin complex	• Optimization of drug release rate by varying copolymer composition and aqueous copolymer concentration. • Significantly lower burst release and controlled zero-order release profile of the system. • Controlled basal insulin delivery *in vitro* and *in vivo* in chemically and structurally stable form.	[15,38,52]
mPEG-PLGA-mPEG	Lysozyme, Salmon calcitonin	• Controlled release of the protein for a longer duration by extending the PLGA block resulting from decreased degradation rate of the copolymer matrix. • Protection of *in vivo* animal model from methylprednisolone acetate induced osteoporosis for up to 40 days.	[14,53]

1.4.2. Commercial and Investigational Examples

Long-term controlled delivery of hydrophilic and hydrophobic drug substances via the parenteral route is an attractive approach. PLA and PEG diblock and triblock copolymers are profoundly investigated for this purpose due to their myriad of benefits. Ensuing a great deal of success in veterinary medication,[55] there is abundant appreciation of the potential for various applications of these copolymers. Substantial investigations are being carried out and several of them have made it to clinical trials.

A sterile, lyophilized micellar formulation of paclitaxel Genexol®-PM (Cynviloq™) using PLA-PEG diblock copolymer has been approved by the Food and Drug Administration (FDA) to be marketed in Europe and Korea.[56] In this copolymeric colloidal carrier PEG served as a non-immunogenic outer shell while the PLA in the hydrophobic core solubilized the hydrophobic drug. The maximum tolerated dose (MTD) of paclitaxel and its biodistribution in liver, spleen, kidneys, lungs, heart, and tumor were both found to be increased by 2-3 folds in preclinical studies. The antitumor efficacy was significantly improved compared to free paclitaxel. Clinical studies have demonstrated better safety profile, higher efficacy, and better response rates of Genexol®-PM in patients with metastatic breast cancer and advanced pancreatic cancer. Combination chemotherapy of Genexol®-PM with cisplatin allowed for the administration of higher doses of paclitaxel and showed significant results. Genexol®-PM also increased response rates for patients who were not responsive to conventional paclitaxel therapy. It is also considered a potentially effective treatment alternative for gemcitabine-resistant pancreatic ductal adenocarcinoma based on promising *in vivo* data. Genexol®-PM has completed Phase I/ phase II trials as a treatment strategy in metastatic breast cancer, non-small cell lung carcinoma, pancreatic cancer, ovarian cancer, and bladder cancer. Studies are under progress for

the treatment of several other diseases, as well as phase III and phase IV studies in recurring breast cancer patients.[57]

Additionally, a paclitaxel incorporated PLGA–PEG–PLGA triblock copolymeric formulation based on MacroMed's proprietary ReGel® technology, called OncoGel™, was investigated for local tumor management. ReGel® is a water-soluble thermosensitive copolymer designed to undergo reversible phase transition from an injectable low viscosity solution incorporating drug of choice (sol-state) between 2 to 15 °C, to a controlled release gel depot at physiological temperature (37 ˚C) 58. Phase I clinical trials of OncoGel™ on patients with inoperable solid tumors was with mixed results. Paclitaxel dose of up to 2.0 mg/cm^3 was well tolerated and the drug remained localized at the injection site. However, pain, injection site bruising, redness, irritation, muscle spasm, and post-procedural discharge were observed as major side effects. In another study, OncoGel™ demonstrated disappointing results in a phase IIb designed model to determine its impact on presurgical potential in patients with esophageal cancer.[57,58] Alongside, a Phase I/II dose escalation study for local injection of OncoGel™ in patients having recurring glioma was terminated within 8 weeks owing to dose-limiting toxicities with serious vascular adverse effects mainly subdural hematoma.[59,60]

1.4.3. Limitations of Thermosensitive Polymers

Thermosensitive copolymers are simple and elegant drug delivery systems which are easy to formulate and on administration at body temperature show instantaneous sol-gel transition. Nonetheless, it is advised to keep the aqueous copolymer solution at 4 ˚C to maintain good injectability and low viscosity of the system as viscosity of the system generally tends to increase as the temperature approaches room temperature. This may be a possible hurdle that will need discretion while taking such drug delivery systems to a clinical setting.[61,62] Alongside,

drugs with high water solubility and small size readily diffuse from these copolymer matrices because of the highly porous microstructure, low degree of crosslinking, and increased hydration and swelling of these copolymers. Studies have also reported that an initial burst release occurs owing to the drug located close to the surface of these copolymeric delivery systems.[16] Numerous studies have also reported irreversible protein aggregation in these delivery systems resulting in incomplete protein release *in vitro* and less than 100% bioavailability *in vivo*. Though, this issue can be resolved to some extent by decreasing the drug loading.[61] Furthermore, the addition PEG has shown to limit the encapsulation efficiency of various drugs and proteins, even when adopting the most pertinent formulation techniques. This effect is suspected to be an effect of steric interference and possible drug/protein-polymer interactions.[12]

1.5. Resorbable Phase Sensitive Polymers

Smart (stimuli-responsive, environmentally-sensitive) polymers offer a drug delivery platform that can be utilized to deliver the drug molecules at a controlled rate and in a stable and biologically active form. [2,17] The uniqueness of smart polymers lies in their nonlinear response triggered by a very small stimulus which causes a significant macroscopic alteration in their structure and properties. The attractiveness of smart polymer-based delivery systems is enhanced by their features such as reduced dosing frequency, improved safety profile, and therapeutic effectiveness. The major advantages of smart polymer-based injectable delivery systems include ease of preparation and administration, prolonged release of incorporated drug, maintenance of desired drug-therapeutic levels with a single dose, site specific/localized delivery, reduced side-effects, increased stability of incorporated drugs during formulation, storage and release, and the most importantly, improved patient compliance and reduction in follow-up care. [17]

Phase sensitive smart polymers allow for easy subcutaneous injection after drug loading. Following injection, the biocompatible, organic solvent is displaced, leaving the therapeutic entrapped and protected within the polymer depot. Its release becomes dependent upon diffusion and breakdown of the polymer over an extended period of time. The ability to adjust the polymer composition as well as the solvent composition allows this smart polymer to be very versatile and easily manipulated to provide the best release profile for the therapeutic of interest.

PLA and PGA, along with their copolymers and other polymers such as PEG, N-stearoyl L-alanine methyl ester (SAM) offer many options for creating the release system needed for a particular drug molecule.[18] By varying the molecular weight and/or composition of the PLA, PGA, or PLGA, the hydrophobicity can be altered and changes in release profile observed. In addition, the solvent composition is also very versatile which will also contribute to the release profile observed.[17,2114,18,34] Common biocompatible, organic solvents used are benzyl benzoate, benzyl alcohol, N-methyl-2-pyrrolidone (NMP), safflower oil, etc. Each organic solvent has a different level of hydrophobicity. For example, benzyl benzoate and benzyl alcohol have different hydrophobicity with benzyl benzoate being more hydrophobic. These solvents can be used alone or in combination to alter how quickly dispersion of the solvent occurs. The dispersion of the organic solvent will influence how the polymer depot forms which can affect the release profile of the drug incorporated. Other factors such as drug size, hydrophobicity, etc. will also influence the release profile. If considered together, the phase sensitive delivery system can be optimized to desired specification.[18,63]

1.5.1. Phase Sensitive Polymer-Based Drug Delivery Systems

Phase sensitive delivery systems hold a lot of potential for controlled delivery. The ease of drug incorporation and versatile nature make phase sensitive delivery systems very appealing.

Optimization is therefore a major tool to develop a delivery system to meet the needs of the drug of interest. Altering the polymer composition, solvent composition, or both will lengthen or shorten the release of incorporated drug. Optimization of polymer and/or organic solvent can also be useful in limiting the burst release which often can be high with controlled delivery systems and also harmful depending on the drug incorporated.

Additional ways to optimize release profile include altering the polymer concentration, the drug concentration, and the injection volume.[18,63] Altering polymer concentration, just like altering composition to change hydrophobicity, will have a big impact on release profile. The higher the polymer concentration, the denser the polymer depot that forms will be after phase transition when organic solvent disperses. The dense polymer matrix will make it more difficult for hydrolysis of polymer and more difficult for drug molecules to diffuse which together will slow release of incorporated drug to alter the release profile. Altering drug concentration can have similar or opposite effects based on the hydrophobic/hydrophilic nature of the drug molecule. As is to be expected, raising the concentration of a hydrophilic drug can decrease the overall hydrophobicity of the release system. This will impact the release profile in a couple of ways such as aiding in hydrolysis of polymer and increasing diffusion of drug through the matrix. Hydrophobic drugs will typically increase the hydrophobic nature of the release system similar to increasing polymer concentration or hydrophobicity but should be carefully evaluated as there is a point where drug that are very hydrophobic can be displaced along with the organic solvent during phase transition. Injection volume can be used as a tool to alter release profile as well. It can be expected that the larger the injection volume, the larger the depot that forms upon phase transition. Increasing the distance a drug molecule must diffuse to be release can slow the release rate and alter the release profile. In cases such as this there may also be a biphasic release

profile in which the drug closest to the surface releases first followed by a plateau before a second release occurs following polymer breakdown and diffusion of drug from within the core of the matrix.[14,53,63,64]

1.5.2. Commercial and Investigational Examples

There have been many investigational examples of phase sensitive release systems. They offer insight to the factors that influence release profile as described above. In addition, they show the extensive options available for developing delivery systems for numerous therapeutics for numerous disease states. The variation seen in the few selected examples in this section will provide a look at the promising future for continued investigation and research into this smart polymer delivery system. Also, a couple of great examples of commercial success can be seen with Eligard® and Atridox®.

In a study by Vintiloiu et al. the potential of a phase sensitive oleogel in situ forming implant was evaluated for the controlled release of rivastigmine to treat Alzheimer's disease.[63] The in situ- forming implant consisted of 5-10% (w/w) N-stearoyl L-alanine methyl ester (SAM) in safflower oil. Burst release of rivastigmine was less than 15% in the first 24 hours and release lasted for 11 days. This formulation development shows potential in extending the dosing interval of rivastigmine while improving upon the benefits of rivastigmine treatment. The process of making this type of formulation is quite extensive and doesn't seem to have the ease of formulation that other in situ- forming implants have. There is also limited optimization that can be done since the authors' found that the rate limiting release mechanism of diffusion through the oily matrix is generally unaffected by the amount of organogelator within the formulation and the density of the gel resulting from the increasing amount of organogelator. Furthermore, the authors failed to examine the biological activity of released rivastigmine and

while they didn't see inflammation at the injection site, no histological analysis seems to have been performed.[65]

Another attempt at controlled release of rivastigmine for the treatment of Alzheimer's disease using an organogel was done by Bastiat et al.[64] N-behenoyl L-tyrosine methyl ester (BTM) in safflower oil showed superior ability in reducing C_{max} when compared to SAM but did not prolong rivastigmine release as release was only for 7 days. BTM controlled burst release to a better extent than SAM and it was also found that implant volume had a greater impact on release kinetics than rivastigmine concentration. This again limits the amount of optimization that could be done to further improve the release profile or offer applications for controlled delivery of other therapeutics.[66] Bastiat et al. further explored tyrosine-based rivastigmine-loaded organogels. A 25 mg/kg *in vivo* study showed release for 14 days when implant volume was 300 µl and increased to 35 days when implant volume increased to 500 µl.[67]

In a study conducted by Ahmed et al.[68] the drug atorvastatin was investigated for its potential in a controlled release, phase sensitive delivery system composed of PLGA and PEG in N-methyl-2-pyrrolidone (NMP). This study does an excellent job of investigating the factors that contribute to release profile as described above. Not only do they look at concentrations of polymers in the composition of the delivery system, but they also looked at composition of PEG used in the delivery system and the impact it has on release profile. These variables selected all relate to hydrophobic nature of the phase sensitive system and nicely demonstrate how the hydrophobic/hydrophilic balance dictates the characteristics of the depot. Ahmed et al. specifically made formulations that varied in PLGA concentration at 20, 30, and 40%, molecular weight of PEG at low, medium, and high molecular weight, and PEG concentrations at 5, 10, and 15%. Using those variables, the team was able to produce 16 formulations to primarily evaluate

effects on burst release at 2 and 24 hrs. *In vitro* they found that PLGA concentration and PEG molecular weight have the greatest impacts on burst release. As is expected, the higher PLGA concentration and higher molecular weight of PEG produced lower burst release and the optimal combination is with the highest tested values of both at 36.10% PLGA and PEG 6000 at 15.69%. *In vivo* the optimized phase sensitive formulation outperformed the oral formulation and phase sensitive formulation that did not contain PEG. They found the plasma concentration values to be 547.62 at 12 hrs, 367.47 at 48 hrs, and 346.84 ng/mL at 72 hrs after oral tablet formulation and intramuscular injection of both phase sensitive formulations, without PEG and optimized formulation, respectively. Furthermore, the mean residence times and t_{max} increased from oral formulation to PEG-free formulation to optimized formulation at about 41, 63 and 80 hours for mean residence time and 12, 48, and 72 hours for t_{max}, respectively. They attributed the differences in phase sensitive formulations to the alteration of glass transition temperature upon addition of PEG and an improved surface smoothness of the depot. They also attributed the difference in release to the faster dissipation of NMP from the formulation that did not contain PEG. That difference in dissolution time is due to the difference in hydrophobicity of the polymer depot where inclusion of PEG slows dissipation of NMP that contains atorvastatin and provides a longer duration of release.[68]

The Atrigel® delivery system was developed in 1987 and is used commercially. Atrigel® is generally comprised of poly (dl-lactide), lactide/glycolide copolymers, and lactide/caprolactone copolymers ranging from 10 to 80% by weight.[69] A range of solvents can be used depending on the hydrophilic to hydrophobic nature desired including dimethyl sulfoxide, N-methyl-2-pyrrolidone (NMP), tetraglycol, glycol furol, propylene carbonate, triacetin, ethyl

acetate, and benzyl benzoate. In addition, certain solvents may be preferred due to the biological effects it can produce.[69]

Eligard® is a commercial phase sensitive formulation used to treat advanced prostate cancer, breast cancer, endometriosis, uterine fibroids, and early onset of puberty. The controlled release of the active ingredient, leuprolide acetate, is available for delivery over 1, 3, 4, or 6 months. [70,71] The release system is composed of the Atrigel® delivery system described above, specifically containing PLGA (50:50; 46000 MW) in N-methyl-2-pyrrolidone (NMP).

Atridox® is another commercial phase sensitive formulation that also employs the Atrigel® delivery system. [72,73] Atridox® is used to treat chronic adult periodontitis. The active ingredient is doxycycline hyclate, but the Atrigel® delivery system specifically uses 36.7% PLA in 63.3% N-methyl-2-pyrrolidone (NMP). The delivery of this antibiotic is maintained over 21 days when locally applied to periodontal pockets to kill bacteria that cause infection.

In addition to the previous commercial formulations that are used to treat disease conditions, there is ATRISORB® FreeFlow™ Bioabsorbable Guided Tissue Regeneration (GTR) Barrier.[74,75] This phase sensitive formulation does not contain an active ingredient, but is used instead, as an isolator and barrier to promote tissue regeneration at the oral, surgical site from the adjacent gingival connective tissue and epithelium following grafting of bone/bone replacement graft material.[76] In addition, a formulation that does contain an active ingredient of 4% doxycycline is also available to provide the same GTR as well as antibiotic treatment.[74,75]

1.5.3. Limitations of Phase Sensitive Polymers

Since phase sensitive delivery systems rely on organic solvents, the application for biologics such as proteins can be a major limitation. Activity of protein therapeutics is only possible if the protein is in its native conformation. Protein environments are aqueous and

therefore exposure to hydrophobic environments alters their conformation and can render them inactive. Short peptides that perhaps do not rely on tertiary structure are still candidates for use in these systems since their activity is not necessarily dependent on conformational structure.

Another limitation of a phase sensitive delivery system is how dependent the release profile is on the nature of the molecule being incorporated. A molecule that is very hydrophilic will not likely go into solution given the hydrophobicity of the organic solvent. Instead, a suspension will be necessary. On the other hand, if the molecule is very hydrophobic it may well disperse with the organic solvent causing a drastic burst release and not entrapping drug in the remaining polymer depot.

Organic solvents are typically avoided due to their toxicity. Therefore, the organic solvents used in phase sensitive delivery systems must be carefully examined for biocompatibility. This will limit the options for solvent choice, but a system that is not well tolerated *in vivo* is essentially of no use.

1.6. Conclusions and Future Perspectives

PLA and PGA based polymers have been shown as excellent drug/protein delivery carriers for easy administration and controlled drug delivery. The biodegradability and biocompatibility of these copolymer systems has attracted a lot of attention over the years for their wide application optimized for the delivery of both small and large molecules with good safety profile.

The striking potential of these copolymeric delivery systems is the ability to be tailored in relation to the therapeutic incorporated, by increasing or decreasing the hydrophilic/hydrophobic ratio resulting in accelerated or decelerated degradation for shorter or longer duration of drug release and can be further exploited. For longer drug release periods synthesizing a polymer with

high degrees of crystallinity can also be considered. Additionally, chemical alterations in the copolymer backbone can be explored for polyelectrolyte complex formation with charged drug molecules in a way to modify the release pattern or enhance the stability of the delivery system. Incorporation of additives can be potentially tested with the delivery systems for their effect on drug delivery or for a combination therapy approach.[20,38]

Overall, these copolymers can be formulated into carriers at multiple scales such as depots, microspheres, nanoparticles, as well as implants. These systems have the ability to incorporate wide range of therapeutics of diversified intrinsic characteristics for their controlled delivery in a chemically and structurally stable form over varying time periods with different possible routes of administration.[12] Further studies will be effective in making this delivery system an ideal approach to administer a large number of protein and peptide based drugs at a controlled rate for longer duration.

1.7. Osteoporosis

1.7.1. Disease Overview

Worldwide, one in three women and one in five men are at risk of an osteoporotic fracture. In US adult population of age 50 years and older, osteoporosis and low bone mass affect approximately 53.6 million people (54% of the population).[77] In addition to considerable pain and disability, osteoporotic bone fractures take a huge personal and economic toll on a person and their family. Elderly patients can develop pneumonia and pulmonary embolism due to prolonged bed rest following a painful fracture.[78] Certain medications such as steroids, anticonvulsants, anticoagulants, antimetabolites, proton-pump inhibitors, thiazolidinediones, and L-thyroxine have also been associated with increased risk of osteoporosis by different mechanisms.[79–85] Osteoporosis, no matter the cause, is a disease of which silent and progressive

loss of bone tissue greatly reduces the density and quality of bones. Consequently, the bones become more porous and fragile with an increased susceptibility to painful fractures resulting in substantial morbidity. The most common fractures associated with osteoporosis occur at the hip, spine and wrist.[86,87] Risk factors chiefly include genetics, old age (>50 years), menopause, low body weight, family history of osteoporosis, history of fracture as an adult, history of hormone and autoimmune disorders, inactive lifestyle, lack of calcium and vitamin D, cigarette smoking, and excessive alcohol consumption.[88]

1.7.2. Current Treatment Methods and Medications

Antiresorptive drugs such as bisphosphonates are most commonly used in clinical practice. Bisphosphonates inhibit osteoclastic bone removal thus increasing bone density, however, use of these drugs is associated with severe acute and long-term side effects which limit their long-term use and patient compliance.[89] Newer treatments such as anabolic synthetic parathyroid hormone, teriparatide and novel antiresorptive antibodies such as denosumab, have been used to increase osteoblast activity (promote new bone growth) and reduce osteoclast activity (inhibit bone resorption), respectively. However, these drugs are expensive and are generally reserved for people with severe osteoporosis who have poor tolerance for other treatments.[90]

Calcitonin is an antiresorptive hormone naturally produced by the parafollicular cells of thyroid gland. It is involved in calcium and phosphorus metabolism and shows a calcium-lowering effect by counteracting parathyroid hormone (PTH).[91,92] PTH acts to increase the concentration of calcium in blood owing to increased bone resorption by altering gene expression in osteoblasts. In bones, calcitonin almost exclusively targets calcitonin receptors on osteoclasts interfering with their differentiation from precursor cells, reducing motility and

inducing retraction by multiple inhibitory mechanisms.[91,93] Calcitonin is frequently used in the treatment of several bone-related disorders such as hypercalcemia, Paget's disease and osteoporosis. In osteoporosis, calcitonin reduces bone resorption and significantly reduces bone pain, a very common symptom of osteoporosis.[94,95] Clinically, synthetic or recombinant salmon calcitonin (sCT) is widely used since it has 50% sequence homology to human calcitonin, meanwhile demonstrating 40 - 50 times higher potency than human calcitonin due to its higher affinity towards human calcitonin receptor.[96,97]

In practice, calcitonin or sCT is administered by subcutaneous, intramuscular, or intranasal routes, daily or multiple times per week, depending on the severity of bone loss.[98–100] However, frequent administration produces discomfort and reduces patient compliance which negatively affects treatment adherence, thus resulting in treatment gaps. [101–103] Calcitonin can be administered through the nasal route, however its bioavailability is only ~25% compared to intramuscular calcitonin.[104] Additionally, intranasal calcitonin is associated with the risk of nosebleeds, runny nose, and other nasal irritations.[31] Oral formulations have been tested, but results demonstrate compromised bioactivity owing to degradation by enzymes within the digestive tract.[32]

1.7.3. Novel Formulations of Salmon Calcitonin

There have been numerous studies aimed at overcoming the downfalls of delivering a therapeutic such as calcitonin. Most of the formulations have interest in primarily protecting salmon calcitonin from degradation in order to preserve its activity. As a secondary interest, controlled release over an extended period of time. The following is simply a minimal selection of examples.

1.7.3.1. Nanoparticles and Beads

One of the first explorations of chitosan beads for the delivery of sCT was by Aydin and Akbuga.[106] In the study, salmon calcitonin was dissolved in a solution containing chitosan in 1.5% acetic acid. Beads formed upon dripping through a glass syringe into tripolyphosphate, pH 6. Encapsulation efficiency was 54-59% and size of beads was ~9 mm in diameter. In vitro release showed a burst release of ~20% and ~27 days for complete release.

In an effort to overcome challenges of oral dosing route, Alonso, et al. investigated surface-modified lipid nanoparticles.[107] The core of the nanoparticles was either a solid triglyceride (tripalmitin), or a mixture of a liquid and a solid triglyceride (Miglyol® 812 and tripalmitin). The surface of these nanoparticles was modified with either chitosan or PEG which ultimately had an effect on the association of sCT with the core lipid. Chitosan was observed to displace sCT more than PEG and therefore a reduced burst release was found when testing release profiles of the nanoparticles. Under in vitro release conditions, release of sCT from the nanoparticles was slow and attributed to the affinity of sCT for the lipid core while lacking a degradation of the lipid.

1.7.3.2. Hydrogels and Smart Polymers

As an example of a hydrogel investigated for the delivery of sCT, there is the study by Basan, Hasan, Gümüşderelioğlu, Menemşe, and Tevfik Orbey, M.[108] This study was done using a biodegradable dextran hydrogel composed of dextran (T-70) crosslinked with epichlorohydrin (ECH). The colon specific *in vitro* release of sCT was carried out in simulated gastrointestinal fluid. Over the course of 17 hrs, ~85% of sCT was released which is partially due to the use of ethanol to reduce swelling of the hydrogel.

Singh et. al. have previously explored variations of thermosensitive triblock copolymers including PLGA-PEG-PLGA and mPEG-PLGA-mPEG with lactide to glycolide ratios up to 3:1 for controlled release of model peptide based therapeutics such as lysozyme and sCT.[14,17,109,110] These previous studies were the basis for exploring lactide to glycolide ratios of 3.5:1, 4.5:1, and 5:1. Initial studies of mPEG-PLGA-mPEG consisted of eleven variations of mPEG-PLGA-mPEG synthesized with serially increasing length of mPEG and PLGA blocks with lactide to glycolide ratios up to 3:1, in order to find a copolymer with the longest hydrophobic PLGA block, while maintaining the desired properties of minimal burst release, controlled release, and complete release of conformationally stable therapeutic.[14] Eleven copolymers were synthesized out of which only four were able to transition from solution to gel form at body temperature, as tested using the test tube inversion method. These were further tested for controlled release and biocompatibility. The release of lysozyme showed the importance of block length in a number of ways.[19] First, it showed how copolymers with smaller mPEG length were able to form more stable gels with lower burst release as well as volume contraction upon expulsion of aqueous phase and push out effect. Second, the larger PLGA block gave insight into its role in slowing degradation of the copolymer which in turn slows release of therapeutic. Larger PLGA block makes the gel more hydrophobic making breakdown, which is primarily due to hydrolysis, more difficult. From this initial study, further testing using sCT was explored in this research using the insight gained.[53] Both lysozyme and sCT retained bioactivity demonstrating the ability of the copolymer to protect the structure of sensitive protein and peptide-based therapeutics. Release of therapeutics was observed over the course of 28 and 42 days for lysozyme and sCT, respectively. Burst release was minimized to ~22% for lysozyme and ~6% for sCT. The complete details and

further insight into the rationale of the current work can be found in those previous publication.[14,17,109,110]

1.8. Alzheimer's Disease

1.8.1. Disease Overview

Alzheimer's disease is the most common form of dementia associated with aging. It is estimated that more than 5 million Americans have Alzheimer's with an annual cost of over $200 million.[111] Alzheimer's disease always results in death and is one of the leading causes of death in America, usually just behind heart disease and cancer. Yet, Alzheimer's disease has significantly less money going towards research and hasn't had as much breakthrough research towards finding treatments or cures, perhaps due to this lack of research dollars.[112,113]

Alzheimer's disease is typically diagnosed upon development of memory loss and cognitive impairment, but the damage to the brain that causes these symptoms usually begins accumulating many years before any indications of disease state.[114] Damage to the brain is caused when proteins aggregate and form amyloid plaques and neurofibrillary tangles. The plaques and tangles lead to neuron cell death and reduction in an extremely important neurotransmitter, acetylcholine. As cell death continues and the disease progresses, the neurons within the hippocampus responsible for things such as memory can no longer transmit proper signals and symptoms become apparent.[115] More damage will lead to worsening symptoms that eventually lead to institutionalization in most cases which accounts for the majority of the cost associated with this disease.[116] The patient will lose their ability to perform daily tasks and usually end up bed ridden prior to death as the body shuts down.[111]

1.8.2. Alzheimer's Disease Medications

While there isn't a cure for Alzheimer's disease, there are some treatment options which are generally prescribed based on the stage of disease progression and typically aim to alter neurotransmitter levels within the brain to alleviate symptoms, table 2 and table 3. Even though treatments ease the symptoms and can allow the patient to remain independent longer, the disease continues to progress. Cholinesterase inhibitors, more specifically acetylcholinesterase inhibitors, are the most common forms of treatment and work by inhibiting enzymatic breakdown of acetylcholine. Most drug therapies are primarily orally administered and are accompanied with side effects including nausea, vomiting, muscle cramps, loss of appetite, etc. Tacrine is no longer typically used in treating Alzheimer's but was the first acetylcholinesterase inhibitor used.[115,117] Donepezil, brand name Aricept, is an acetylcholinesterase inhibiting treatment option for mild to severe Alzheimer's.[118] Galantamine, brand name Razadyne, is another cholinesterase inhibitor used to treat mild to moderate Alzheimer's. In addition to preventing acetylcholine breakdown, there is also nicotinic stimulation in an effort to increase acetylcholine release in the brain.

Memantine, brand name Namenda, is a treatment option for moderate Alzheimer's. As a NMDA (N-methyl D-aspartate) antagonist, memantine works by preventing toxic effects of excess glutamate by regulating glutamate activation. Gastrointestinal upset, dizziness, confusion, and headache are common side effects.[118] Namzaric is the brand name of a combination therapy involving memantine extended release along with donepezil. The mechanism of action combines those of both drugs and therefore provides the symptom relief associated with decreased toxic effects of glutamate and increased acetylcholine. A capsule

formulation is available to treat moderate to severe Alzheimer's with the common side effects including those from the individual drugs.[118]

Rivastigmine, brand name Exelon, works in a similar fashion to donepezil as a cholinesterase inhibitor, but until recently it hadn't been approved to treat severe Alzheimer's disease and was only prescribed for mild to moderate Alzheimer's disease.[119] Rivastigmine is unique in that it inhibits acetylcholinesterase as well as butyrylcholinesterase. In addition, it has more formulation options including a transdermal patch which can actually alleviate some of the gastrointestinal side effects.[118,120–124] In the following sections we will look at the background of rivastigmine along with an in-depth view of the mechanism of action. Next, the conventional formulations commercially available will be explored and the shortcomings considered. In addition, novel approaches to overcome conventional formulation shortcomings shall be presented and analyzed. Finally, future directions for pursuing optimal treatments will be discussed.

Table 2. Summary of Alzheimer's Medications and Corresponding Mechanism of Action.

Alzheimer's Medication	Mechanism of Action
Tacrine (Cognex)	Acetylcholinesterase Inhibitor
Donepezil (Aricept)	Acetylcholinesterase Inhibitor
Rivastigmine (Exelon)	Acetylcholinesterase and Butyryl cholinesterase Inhibitor
Memantine (Namenda)	Glutamate Activation Regulator
Memantine + Donepezil (Namzaric)	Cholinesterase Inhibitor and Glutamate Activation Regulator
Galantamine (Razadyne)	Acetylcholinesterase Inhibitor and Nicotinic Receptor Stimulator

Table 3. Summary of Alzheimer's Medications and Corresponding Formulation Availability.

Alzheimer's Medication	Available Formulations					
	Oral Solution	Oral Capsule	Oral Tablet	Oral Extended Release Capsule	Orally Disintegrating Tablet	Transdermal Patch
Tacrine (Cognex)	Discontinued					
Donepezil (Aricept)			✓		✓	
Rivastigmine (Exelon)	✓	✓				✓
Memantine (Namenda)	✓		✓	✓		
Memantine + Donepezil (Namzaric)		✓				
Galantamine (Razadyne)	✓		✓	✓		

1.8.3. Rivastigmine: Background and Mechanism of Action

Marta Weinstock-Rosin was working to find a drug similar to morphine that wouldn't cause respiratory depression when she and other chemists at Hebrew University's Department of Pharmacology developed a semi-synthetic derivative of physostigmine, Exelon. It did not work in the intended but in the front part of the brain. The drug was then sold by Yissum to Novartis and commercial development lead to capsule and liquid formulations in 1997 and a patch formulation in 2007.

The main mechanism of action of Rivastigmine is the prevention of acetylcholine metabolism by binding to the esteratic and ionic sites on acetylcholinesterase. The binding of rivastigmine is preferential for the G1 isoform of acetylcholinesterase which is the dominant form in patients with Alzheimer's.[125,126] Rivastigmine largely acts on cholinesterase within the central nervous system, thus preventing increased acetylcholine concentrations throughout the rest of the body which would otherwise cause many complications as acetylcholine is an extremely common neurotransmitter.

The mechanism of action has great advantages over other treatment options in addition to many other positive aspects. Being the only treatment option with a patch formulation, rivastigmine allows for increased dosing interval, better patient compliance, and decreased caregiver burden.[103,127–130] Treatment in the early stages of Alzheimer's disease will allow the patient to remain independent longer and delay institutionalization.[116,131] This is significant since the majority of the cost of the disease arises due to institutionalization. In addition, this too relieves and delays caregiver burden.[103,129] As for advanced Alzheimer's disease, there is support showing rivastigmine can help with eating problems.[132]

1.8.4. Conventional Rivastigmine Formulations

Oral formulations were the first treatment options available for rivastigmine in the treatment of Alzheimer's Disease. Oral solution is available as a 2 mg/ml rivastigmine tartrate equivalent dose to rivastigmine base. Daily administration of 6-12 mg should be divided into two doses and should be taken with food. An advantage of this formulation is the ability to mix it with water or juice, etc. to make administration more convenient. Oral capsules are another available formulation and can be interchanged with oral solution at the same dose.[119]

Transdermal patch formulation is what sets this treatment option apart from other Alzheimer's medications. The transdermal patch was designed to increase the dosing interval and provide an easier, more convenient method of administration. It can ease compliance issues by reducing the times per day medication must be taken and avoids the GI tract to prevent common negative side effects seen with oral administration. In addition, it maintains therapeutic drug levels within the body better than oral formulations to maximize the symptom relief this drug can offer patients.[119]

1.8.5. Shortcomings of Conventional Rivastigmine Formulations

As with any medication, there are some drawbacks to different rivastigmine formulations. First, the oral formulations cause a lot of negative gastrointestinal symptoms, such as stomach upset, vomiting, diarrhea, etc. This was one of the key factors when developing the patch formulation in addition to increasing the dosing interval. The patch can bypass the gastrointestinal system all together to prevent those adverse reactions while avoiding first pass metabolism in the liver. However, the patch can cause skin irritation with an incidence of hemorrhagic bullous lesion.[129–131,133] Second, the patch has variability in release between anatomical positions as well as between people. This can also be a factor in the oral formulations as intestinal absorption may vary as well. Hence, the third pitfall is the wasted drug in any formulation. Only 10-50% of the drug within a patch is released and for oral formulations only about 40% becomes bioavailable.[119] Another important thing to consider in this patient group is overdose due to memory loss. Multiple patches or doses of oral rivastigmine can lead to accidental overdose.[134] On the contrary, it can also be the cause of noncompliance in these patients.[103] Aside from forgetting to take the medication, dosing multiple times per day as with the oral formulations or even daily with the patch can be inconvenient and causes rise and fall of drug levels within the body. The main benefit of rivastigmine treatment is the relief of symptoms, but this benefit is only optimal when a constant level of tolerated drug is maintained in the body which is not the case when dosing intervals are frequent. All of these limitations produce a need for more research and development of better dosing options.

1.8.6. Novel Approaches to Resolve Conventional Formulation Shortcomings

Ideally, a formulation that can control and maintain the release over a prolonged period of time with little variability between patients is the goal. In addition, the formulation should be

biocompatible and provide complete release of rivastigmine while avoiding first pass metabolism.

Many delivery methods and drug combinations have been researched as potential treatment options. Each of these methods have shown promise, but none have made it to the market to date. The main focus of each is generally to provide a longer dosing interval and maintained therapeutic levels of rivastigmine to maximize symptom relief. As outlined below, each method or combination has its merits and perhaps some limitations.

1.8.6.1. Rivastigmine Hybrids

In a recent study by Li et al. rivastigmine was used as a model for the development of new hybrids with curcumin as site-activated multitarget-directed ligands.[135] A series of novel 2-methoxy-phenyl dimethyl-carbamate compounds showed commendable acetylcholinesterase and butyrylcholinesterase activity in addition to beta-amyloid aggregation inhibition, radical cation scavenging and metal chelating activity. The most potent hybrid explored in this study was approximately 20 times more potent than rivastigmine with an IC_{50} value of 0.097 μM. Beta-amyloid fibril formation was shown to be inhibited when examined by transmission electron microscopy. The combined effects show promise for Alzheimer treatment where cholinesterase inhibition is coupled with inhibition of Aβ-aggregation due to a potentially important role of ortho-methoxy carbamate moiety in binding to beta-amyloid.[135]

In another attempt to improve Alzheimer's treatment using hybrid technology, Babitha et al. found a promising candidate, p-chlorophenyl substituted rivastigmine and fluoxetine hybrid compound.[136] The pharmacological profile indicated electrostatic interactions and hydrogen bonding that is superior, and its high acetylcholinesterase activity could better address a narrow therapeutic window associated with acetylcholinesterase treatment. However, the binding,

biological activity, and overall ADMET were only calculated and predicted in silico. The actual characteristics would need to be evaluated *in vitro* and *in vivo* before any major conclusions could be drawn about this hybrid and its potential as a treatment for Alzheimer's disease. As shown throughout the history of pharmaceutical research, many of the most promising candidates often fail before ever making human trials.[136]

Hybrids of rivastigmine-scutellarin were evaluated for their treatment properties such as chelating, neuroprotective, and antioxidant in addition to the cholinesterase inhibition by Sang et al.[137] The multifunctional properties were confirmed *in vitro* and *in vivo* in mice with scopolamine-induced cognitive impairment. The neuroprotective effects were seen *in vivo* and were largely attributed to the scutellarin anti-inflammatory, free radical scavenging, and Aβ fibril formation inhibition properties after crossing the blood brain barrier. Combining the effects of rivastigmine's acetylcholinesterase inhibition with inhibition of Aβ fibril formation again shows promise in treating and preventing more damage. A drawback is that one of the two most promising hybrids didn't have even weak affinity for BuChE. As explained previously, BuChE increases activity as Alzheimer's progresses which negates the authors' suggestion that affinity for only AChE could be beneficial and reinforces why the hybrid with BuChE performed better. In addition, the study failed to detail which isoform of AChE was used in modeling the docking of the hybrid. Again, as explained previously, the isoform of AChE is important because it is the G1 isoform that is most prevalent in Alzheimer's disease patients and to which binding would be most beneficial.[137]

1.8.6.2. In Situ-Forming Implants

In a study by Vintiloiu et al. the potential of an oleogel implant was evaluated.[65] The in situ- forming implant consisted of 5-10% (w/w) N-stearoyl L-alanine methyl ester (SAM) in

safflower oil. Burst release of rivastigmine was less than 15% in the first 24 hours and release lasted for 11 days. This formulation development shows potential in extending the dosing interval of rivastigmine while improving upon the benefits of rivastigmine treatment. The process of making this type of formulation is quite extensive and doesn't seem to have the ease of formulation that other in situ- forming implants have. There is also limited optimization that can be done since the authors' found that the rate limiting release mechanism of diffusion through the oily matrix is generally unaffected by the amount of organogelator within the formulation and the density of the gel resulting from the increasing amount of organogelator. Furthermore, the authors failed to examine the biological activity of released rivastigmine and while they didn't see inflammation at the injection site, no histological analysis seems to have been performed.[65]

Another attempt at controlled release using an organogel was done by Bastiat et al. N-behenoyl L-tyrosine methyl ester (BTM) in safflower oil showed superior ability in reducing C_{max} when compared to SAM but did not prolong rivastigmine release as release was only for 7 days.[66] BTM controlled burst release to a better extent than SAM and it was also found that implant volume had a greater impact on release kinetics than rivastigmine concentration. This again limits the amount of optimization that could be done to further improve the release profile or offer applications for controlled delivery of other therapeutics.[66] Bastiat et al. further explored tyrosine-based rivastigmine-loaded organogels.[67] A 25 mg/kg *in vivo* study showed release for 14 days when implant volume was 300 µl and increased to 35 days when implant volume increased to 500 µl.[67] However, organogels have been known to have inherent toxicity due to their organic phase.

1.8.6.3. Rivastigmine Nanoparticles

Nanoparticles have been widely explored as potential delivery options for rivastigmine. Polymeric nanoparticles can control release of entrapped drug while remaining stable under biological conditions. In addition, their composition can be modified to allow incorporation of targeting moieties. Ranging in size from as small as 10 nm, nanoparticles can provide transport across biological barriers where their payload is finally released via various mechanisms. Based upon hydrophobicity, certain production methods can be employed to optimize entrapment.[138]

Wilson et al. used poly(n-butylcyanoacrylate) (PBCA) nanoparticles coated with polysorbate 80 to increase the amount of rivastigmine that reached the brain by 3.82-fold when compared to free drug. However, Joshi et al. found that when comparing PBCA nanoparticles to PLGA nanoparticles loaded with rivastigmine the higher molecular weight of PLGA nanoparticles appeared to control the release of rivastigmine better than PBCA.[139] While the results of the study by Joshi et al. may undermine the results of Wilson et al., both formulations require intravenous injection which limits administration options for patients, would require frequent dosing, and therefore possibly cause noncompliance.

Rivastigmine loaded L-lactide-depsipeptide polymeric nanoparticles entrapped 60.72 ± 3.72% after optimization by Pagar and Vavia. [140] Particle size was 142.2 ± 21.3 nm and over 72 hours more than 90% of rivastigmine was released *in vitro*. The research group reports a simple method of preparation that allows for optimization by varying factors such as concentration of stabilizer, rivastigmine, and polymer along with sonication time, etc. which they explored to achieve the reported entrapment and circulation time. Entrapment of rivastigmine tartrate is nothing short of difficult due to its hydrophilic nature and small size. Rivastigmine tartrate is also difficult to control the release of in these formulations again because its hydrophilic nature can

aid diffusion to the external aqueous environment. 60% entrapment is very commendable, but the ability to consistently reproduce that entrapment efficiency may be difficult in a pharmaceutical production scenario. Intravenous administration is again a downfall and with only 72 hrs of release, frequent dosing could impact patient compliance and benefit from therapy.[140]

Intranasal delivery of chitosan nanoparticles loaded with rivastigmine was investigated by Fazil et al. [141] The nanoparticles had better brain targeting than rivastigmine solution delivered via intranasal route as well as when delivered intravenously. Intranasal delivery has many benefits especially when the drug's mechanism of action is in the brain. Using chitosan also has benefits because it can be modified to further help targeting and delivery of payload. The entrapment efficiency was ~73%, but the release is less than favorable because it was biphasic and only lasted 24 hrs. Administration route of these nanoparticles would be easier for patients in comparison to intravenous injection, but still frequent and not likely to maintain a constant drug level for maximum symptom relief.[141]

1.8.6.4. Rivastigmine Liposomes

Liposomes have also been explored as a delivery system for rivastigmine. Yang et al. tested liposomes and liposomes coupled with cell penetrating peptide (Gly-Leu-Pro-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg) to increase rivastigmine brain concentration.[142] *In vitro*, both formulations showed enhanced microvasculature permeability of murine blood brain barrier model. *In vivo*, intranasal administration of rivastigmine solution or formulations showed to have better brain penetration than i.v. administration with the cell penetrating peptide coupled liposomes being most effective. Entrapment efficiency was much lower in the liposome formulations without CPP at only ~30%. The duration of absorption and action was significantly

lower as well at only a maximum of 240 minutes for liposomes without CPP as compared to those with CPP.[142]

1.8.7. Future Directions

While more research is desperately needed, the future looks promising. Some interesting advances in Alzheimer's research include an amyloid-β peptide vaccine that could potentially be used to reduce deposition of amyloid within the brain of patients.[143] Another potential treatment could come from research of Cohen et al. who have found that a molecular chaperone can bind to amyloid-β fibrils to prevent further aggregation of amyloid-β and thus reduce toxicity.[144] Detection methods are key to starting treatment as early as possible and correlations between Amyloid-β levels in the cerebral spinal fluid (CSF) and plasma would make detection as easy as a simple blood test.[145] However, CSF levels have not been shown to correlate with disease progression and severity.[146] This could be partially due to the qualitative ways in which cognition is measured from patient to patient and as these methods improve, perhaps we will be able to use biomarkers to gauge how advanced the disease is and what method of treatment is best.[147]

In addition, other research has explored new molecules for treatment options. Quinoline derivatives have been explored as possible inhibitors of amyloid-β accumulation while also able to dissolve plaques that have already formed through chelation.[148] A new molecule, meserine, was synthesized by Shao et al. as a novel carbamate acetylcholinesterase inhibitor. This molecule was based on the opioid meptazinol but given a phenylcarbamate group to confer inhibitory activity.[149] Piperine has shown neuroprotective effects, but lacked promising bioavailability when taken orally. In order to overcome this obstacle Elnaggar et al. developed chitosan nanoparticles loaded with piperine to be delivered intranasally. They found that this

delivery method allowed for piperine to cross the blood brain barrier and convey its antioxidant, neuroprotective activity.[150] Derivatives from compounds such as chalcone, benzo(c) chromen-6-one, and 4-chlorosalicyclic acid have shown potential as cholinesterase inhibitors with similar or better inhibition as current treatment options.[151–153]

Eddy Liew and Nancy Ip found that injecting IL 33 to mice with progressive Alzheimer's like disease produced improved memory and cognitive function similar to healthy mice.[154] Other recent research by Beth Stevens has questioned the way we think about cause and effect in disease progression. Her research supports an idea that synaptic pruning gone awry could be the cause of synapse loss.[155] Additionally, the location of initial tau plaque development has been potentially pinpointed to the locus coeruleus in the brainstem.[114]

Regardless of the therapeutic chosen to treat Alzheimer's Disease, the dosing regimen is going to be a key factor in its success. The therapeutics such as rivastigmine that have proven effective will need to focus on extending the dosing interval and maintaining therapeutic levels of the drug within the body to have greatest impact. These drugs relieve symptoms when at therapeutic doses, but currently there is drastic rise and fall of drug levels between dosing. The result is inefficient symptom relief that may wane. Controlled release delivery systems could be a promising solution to this downfall among current treatment options. Thermosensitive smart polymers in particular have many favorable characteristics such as releasing therapeutics at a steady rate to maintain drug levels at therapeutic ranges. They are solutions at room temperature which makes formulation with one or multiple therapeutics a breeze and only transition into a gel depot at body temperature after subcutaneous injection. Some of the developments emerging in disease prevention and reversal could easily be combined in a thermosensitive formulation with a symptom relief therapeutic such as rivastigmine to offer maximum disease management. Unlike

some other controlled delivery devices, the depots are usually biodegradable and do not require surgery for implantation or removal. In addition, the copolymers are very versatile to allow optimization of release profile, etc. to meet the needs of the therapeutic at hand. Thermosensitive copolymers of PEG and PLGA show praisable potential because they inherently accommodate the needs of optimal controlled release delivery systems: easy to synthesize and optimize, biocompatible, degradable, control release of therapeutic or multiple therapeutics, prolong duration of release and extend dosing interval, provide constant levels of therapeutic for maximum benefits, avoid gastrointestinal side effects, and first pass metabolism by the liver.[14,19,53,156]

1.8.8. Conclusions

With recent research efforts in Alzheimer's disease intervention, the future for a viable, patient compliant therapy is bright. Various treatment options are being explored and more information are being discovered. Rivastigmine is playing an important role in current treatment options and has even more potential as shown by its recent approval for treatment of severe Alzheimer's disease and the ongoing research that is being done. Rivastigmine's powerful symptom relief coupled with a controlled release delivery system such as thermosensitive copolymers that could increase the dosing interval just might be the next way to advance the treatment of Alzheimer's disease.

1.9. Statement of the Problem

Many diseases require frequent dosing due to short activity of the therapeutic. In addition, a complex dosing schedule, inconvenient and uncomfortable dosing routes, and symptoms of the disease being treated can negatively impact patient compliance. In order to overcome short activity of salmon calcitonin and rivastigmine, a complex dosing schedule,

inconvenient and uncomfortable dosing routes, and symptoms of the disease being treated, development of a controlled release delivery system using smart polymers was investigated.

We hypothesized that optimization of thermosensitive and phase sensitive smart polymers will lead to development of a controlled release delivery system for sCT or rivastigmine. Our hypothesis included increasing the lactide to glycolide ratio within the PLGA block of the thermosensitive copolymer to decrease the burst release, provide complete release, and do so with a zero-order release profile. Thermosensitive and phase sensitive polymers were expected to be biocompatible and release incorporated drug in a controlled manner after a single subcutaneous injection, which were tested in vitro and in vivo. Our efforts can potentially significantly improve patient compliance, quality of life, and offer astounding advantages to the current delivery system technologies.

To test our hypothesis, the following specific aims were studied:

1. Synthesis and physicochemical characterization of mPEG-PLGA-mPEG triblock copolymers with increasing lactide to glycolide ratios of 3.5:1, 4.5:1, and 5.0:1 within the PLGA block: Using ring opening polymerization, we synthesized polymers with increased lactide content in the PLGA block to decrease burst release via decreased water absorption and hence, decreased degradation rate. Each polymer was characterized for molecular weight by gel permeation chromatography (GPC). Polymer structure were confirmed using ^{1}H-NMR. Sol-gel transition was determined using the test tube inversion method. MTT assay was conducted to determine biocompatibility in HEK 293 cells.
2. In vitro release studies of sCT (~20 µg/day) from the thermosensitive copolymer system: sCT was mixed with thermosensitive copolymer to fully entrap all therapeutic. Phosphate

buffered saline with 0.02% sodium azide served as the release medium. Samples were kept in a reciprocal water bath at 37 °C and 35 rpm. Micro BCA was used to quantify the amount of sCT released while conformational and chemical stability was analyzed using CD and/or DSC.

3. In vitro formulation optimization of rivastigmine (1.2mg/day) from thermosensitive and phase sensitive systems was performed: rivastigmine was mixed with thermosensitive copolymer to fully entrap all therapeutic. Several formulation parameters were studied and compared including polymer concentration, rivastigmine concentration, polymer composition, solvent composition, depot volume, and rivastigmine hydrophobicity. Phosphate buffered saline with 0.02% sodium azide served as the release medium. Samples were kept in a reciprocal water bath at 37 °C and 35 rpm. HPLC method was used to quantify rivastigmine as well as confirm conformational and chemical stability.
4. Evaluation of *in vivo* biocompatibility and release profile of rivastigmine from the selected polymeric formulation in female Sprague dawley rats was performed: Three experimental conditions which included polymer/rivastigmine formulation, control of rivastigmine aqueous solution, and blank control PBS without rivastigmine were tested. Rivastigmine in plasma obtained from the rats were extracted and analyzed using HPLC to determine rivastigmine concentration at specific time points and also to verify the release of intact rivastigmine. Histological analysis of subcutaneous tissue at the injection site were done to confirm biocompatibility. Acetylcholinesterase activity was analyzed using Ellman's assay at selected time points throughout the study for each animal group.

2. MATERIALS AND METHODS

2.1. Materials

Glycolide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and isophorone diisocyanate were purchased from Sigma Aldrich (St. Louis, MO, USA). D, L-lactide and methoxypolyethylene Glycol-500 were acquired from TCI America (Portland, OR, USA) and Polysciences Inc. (Warrington, PA, USA), respectively. PLGA 50:50 was purchased from Absorbable Polymers International. PLGA 85:15 and PLA (109 kDa) were acquired from Birmingham Polymers Inc., (Pelham, AL, USA). PLA (40 kDa) was acquired from Polyscitech (West Lafayette, IN, USA). Stannous octoate was purchased from Pfaltz and Bauer Inc. (Waterbury, CT, USA). Salmon calcitonin was procured from Calbiochem (Burlington, MA, USA). Micro-bicinchoninic (micro-BCA) protein assay kit was obtained from Pierce Biotechnology Inc. (Rockford, IL, USA). Human embryonic kidney (HEK 293) cell lines, Dulbecco's modified Eagle's medium, and phosphate buffered saline (PBS) were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Rivastigmine tartrate (RT) and rivastigmine base were obtained from TCI America (Portland, OR, USA) and Xi'an Health Biomedical Technology Co. (China), respectively. All other chemicals were of analytical grade and used without further modification. DNTB (5,5'-dithiobis-(2-nitrobenzoic acid) was purchased from Sigma-Aldrich (St. Louis, MO, USA). S-Acetylthiocholine iodide 98% was purchased from Alfa Aesar (Ward Hill, MA, USA). All animal experiments were conducted as approved by the Institutional Animal Care and Use Committee (IACUC) at North Dakota State University (Protocol #A16079). Adult Sprague Dawley (SD) rats were used to evaluate the release profiles and biocompatibility of phase sensitive formulations. The animals were housed

under controlled temperature conditions with 12 h light and dark cycles and were allowed free access to food and water.

2.2 Methods

2.2.1. Synthesis and Characterization of Thermosensitive Triblock Copolymers

Thermosensitive triblock copolymer methoxypolyethylene glycol-polylactide-co-glycolide- methoxypolyethylene glycol (mPEG-PLGA-mPEG) was synthesized using ring opening polymerization following diblock condensation (figure 3).[14] Lactide (LA) to glycolide (GA) ratios were varied in the PLGA block (3.5:1, 4.5:1, and 5:1) to optimize hydrophobic and hydrophilic characteristics of the copolymer. Briefly, mPEG (11g) was dissolved in anhydrous toluene in a three-necked round bottom flask heated to 120 ˚C under continuous stirring. Glycolide (2.32g), lactide (10.08, 12.96, or 14.4g for 3.5:1, 4.5:1, or 5:1, respectively), and catalyst stannous octoate (0.03% w/w) were then added and refluxed under nitrogen atmosphere for 18 h to produce mPEG-PLGA diblocks. The temperature was then cooled to 60 °C and isophorone diisocyanate was added in excess (~7 mL) followed by coupling for 12 h. The contents were then refluxed at 120 ˚C for 6 h followed by cooling to room temperature. The copolymer was then purified by addition of ice-cold anhydrous diethyl ether followed by removal of organic solvent twice. Purified copolymer was vacuum dried to completely evaporate residual organic solvents.

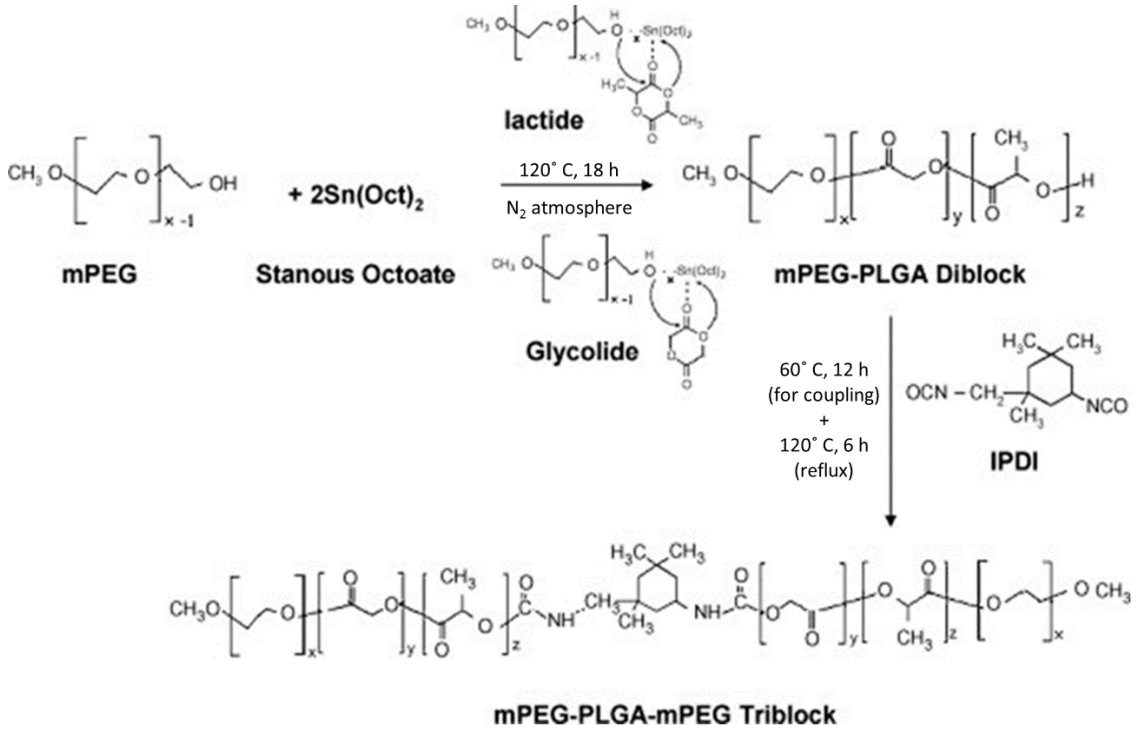

Figure 3. Synthesis of mPEG-PLGA-mPEG via Ring Opening Polymerization.
Note: Schematic of synthesis of triblock copolymer by ring opening polymerization reaction followed by diblock condensation.

2.2.2. Sol to Gel Transition Temperature

Sol to gel transition temperature of polymer with increasing LA:GA was determined using tube-inversion method. Copolymer samples were dissolved in deionized water at 30 and 40% (w/v) concentration and injected into glass tubes immersed in a water bath. Using 2 °C increments, the temperature of the water bath was raised from room temperature (~27 °C) to 43 °C while allowing the samples to acclimatize for 10 min at each temperature point. After 10 min the glass tubes were inverted and the characteristics (sol/gel) of the copolymer was analyzed. The copolymer with physiologically relevant phase transition temperature was further analyzed.

2.2.3. ^{1}H-NMR Spectroscopy

Copolymer sample was dissolved in in $CDCl_3$ and analyzed using ^{1}H-NMR (Mercury Varian 400 MHz) spectroscopy to determine its structural composition. Tetramethylsilane was

taken as the zero-chemical shift. Representative peaks for lactide ($-CH_3$) and glycolide ($-CH_2$) components were integrated to determine lactide to glycolide ratio of the copolymer.

2.2.4. Gel Permeation Chromatography

Copolymer sample (5 mg/mL) was prepared in tetrahydrofuran and analyzed using GPC (Tosoh Bioscience EcoSEC HLC-8320: modular system with refractive index and UV detectors) to find retention time and determine weight average molecular weight, number average molecular weight, and polydispersity index of the synthesized copolymer.

2.2.5. Critical Micelle Concentration

CMC of the copolymer was determined using pyrene as a hydrophobic probe.[157] Pyrene was dissolved in acetone (24 µg/mL) and 10 µL aliquots were added to each glass test tube. Acetone was evaporated, and 2 mL aqueous polymer solution was added to each tube at increasing polymer concentrations ranging from 0.5 to 1000 µg/mL. The test tubes were vortexed briefly multiple times followed by a resting period in the dark for 12 h. Fluorescence of pyrene at each concentration was measured using a Fluoromax-4 spectrofluorometer (Horiba Jobin Yvon, NJ, USA) with excitation set at 336 nm and emission from 360 – 450 nm (excitation and emission slit widths of 1 nm). Intensity ratios of peaks 379 and 393 nm were calculated and plotted against logarithm of concentration to determine CMC.

2.2.6. *In vitro* Biocompatibility Assay

In vitro biocompatibility of the polymer was estimated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assay using human embryonic kidney cell line (HEK-293) and Dulbecco's modified Eagle's medium (DMEM). Cells were seeded in a 96 well plate at a density of 5 $x10^3$ cells per well followed by incubation at 37 °C and 5% CO_2. After 24 h of incubation, the media was replaced with fresh serum-free

media containing increasing concentrations of copolymer (0.1, 1, 2, 5, or 10 mg/mL). Cells were further incubated with the copolymer sample for 24 h. Cells incubated without any copolymer were taken as control. Following incubation, media was removed and 20 µL of MTT solution (5 mg/mL) was added to each well and incubated for 2 h to allow formation of formazan crystals. MTT solution was carefully aspirated and dimethyl sulfoxide (150 µL) was added to each well to dissolve formazan crystals. Absorbance was recorded at 570 nm and relative cell viability was calculated using the following equation: **Relative cell viability (%) = ($A_{polymer}$ / $A_{DMEM\ only}$) x 100**, where, $A_{polymer}$ is average absorbance of wells incubated with polymer samples and $A_{DMEM\ only}$ is the average absorbance of the control wells incubated with serum-free DMEM.

2.2.7. *In vitro* Release Profile of Salmon Calcitonin

Formulations were prepared by suspending sCT (2.5 mg) in 30 and 40% w/v aqueous copolymer solutions. Using a 25 G syringe, 0.5 mL of the formulation being tested was injected in each glass tube and incubated in a water bath at 37 °C to form a gel depot. Pre-warmed PBS (10 mM, pH 7.4) containing 0.02% w/v sodium azide was then added to each tube as release medium (4 mL per tube). The tubes were capped to prevent evaporation and incubated at 37 °C under constant shaking at 35 rpm. Sample aliquots (1 mL) were taken at selected time points and replaced with 1 mL fresh pre-warmed release medium. Released protein sCT was quantified using micro BCA protein assay kit, following manufacturer's instructions. Cumulative percent released was calculated over the course of delivery.

2.2.8. Stability of Salmon Calcitonin

Conformational changes in sCT released from the thermosensitive copolymer formulation were evaluated both at physiological and storage temperatures of 37 ˚C and 4 ˚C, respectively, using circular dichroism (CD) spectroscopy.

2.2.8.1. Stability of Salmon Calcitonin at Physiological Temperature

Conformational stability of sCT released *in vitro* from thermosensitive copolymer formulation mPEG-$PL_{3.5}GA_1$-mPEG (LA:GA, 3.5:1) 40% (w/v), at 37 ˚C was analyzed at specific time intervals using circular CD spectroscopy. Samples were centrifuged, filtered and degassed prior to analysis. PBS (10 mM, pH 7.4) was used as reference buffer. CD spectra were scanned in far-UV region (190 - 230 nm) to investigate the changes in the secondary structure of sCT. All spectra were recorded at a scan rate of 50 nm/min at 20 °C using a quartz cuvette (0.1 cm path length). Freshly prepared sCT solution in PBS (10 mM, pH 7.4) was used as standard. Spectra manager®2 software (Jasco, Tokyo, Japan) was used for spectrum analysis.

2.2.8.2. Stability of Salmon Calcitonin Inside Gel During Storage at 4 °C

Conformational stability of sCT incorporated in thermosensitive copolymer formulation mPEG-$PL_{3.5}GA_1$-mPEG (LA:GA, 3.5:1) 40% (w/v), at storage temperature (4 ˚C) was also determined using CD spectroscopy as aforementioned. sCT was extracted from the copolymer formulations using acetonitrile (ACN)-PBS (1:1, v/v) at predetermined time points.[44] The stability of sCT, as evidenced by the presence of two peaks in ACN-PBS, was compared with that of freshly prepared sCT [1mg/mL, ACN-PBS (1:1, v/v)].

2.2.9. Thermosensitive Formulation Preparation for Comparing Polymer Concentration

Thermosensitive copolymer was dissolved in water at 20-35% w/v. Rivastigmine tartrate or base was incorporated into the homogenous copolymer system at 5 or 20 mg/ml respectively.

2.2.10. Thermosensitive Formulation Preparation for Comparing Drug Concentration

Thermosensitive copolymer was dissolved in water at a 35% (w/v). Rivastigmine tartrate was incorporated into the homogenous copolymer system at concentrations of 5, 18, and 36

mg/ml while rivastigmine base was incorporated into the homogenous copolymer system at concentrations of 5, 10, 20, and 40 mg/ml.

2.2.11. Thermosensitive Formulation Preparation for Comparing Depot Volume

Thermosensitive copolymer was dissolved in water at 35% (w/v). Rivastigmine base was incorporated into the homogenous copolymer system at 20 mg/ml.

2.2.12. Phase Sensitive Formulation Preparation for Comparing Polymer Concentration

Phase sensitive samples were prepared with PLA (109 kDa) at polymer concentrations of 2.5, 5, and 10% (w/v) in 100% benzyl benzoate. Vigorous mixing and sonication allowed for homogenous preparations to be formulated. Rivastigmine base was incorporated at a concentration of 60 mg/ml.

2.2.13. Phase Sensitive Formulation Preparation for Comparing Drug Concentration

Phase sensitive samples were prepared with PLA (109 kDa) at polymer concentration of 5% (w/v) in 100% benzyl benzoate. Vigorous mixing and sonication allowed for homogenous preparations to be formulated followed with the incorporation of rivastigmine base at concentrations of 60, 120, and 180 mg/ml.

2.2.14. Phase Sensitive Formulation Preparation for Comparing Polymer Composition

Polymer selection was examined for four polymers of varying molecular weight and/or composition: PLA (MW:109 kDa), PLA (MW:40 kDa), PLGA (PLA:PGA, 85:15), and PLGA (PLA:PGA, 50:50). Phase sensitive samples were prepared at polymer concentration of 5% (w/v) in 100% benzyl benzoate. Vigorous mixing and sonication allowed for homogenous preparations to be formulated followed with the incorporation of rivastigmine base at 216 mg/ml.

2.2.15. Phase Sensitive Formulation Preparation for Comparing Solvent Composition

Solvent composition was examined for all four aforementioned polymers by varying the ratio of benzyl benzoate (BB) to benzyl alcohol (BA). Phase sensitive samples were prepared at polymer concentration of 5% (w/v) in each of the following benzyl benzoate to benzyl alcohol (BB:BA) solvent compositions: 100:0, 95:5, 90:10, and 85:15. Vigorous mixing and sonication allowed for homogenous preparations to be formulated followed with the incorporation of rivastigmine base at 216 mg/ml.

2.2.16. Phase Sensitive Formulation Preparation for Comparing Drug Hydrophobicity

Phase sensitive samples were prepared with PLGA (50:50) at polymer concentration of 5% (w/v) in 95:5 benzyl benzoate: benzyl alcohol solvent. Vigorous mixing and sonication allowed for homogenous preparations to be formulated followed with the suspension of rivastigmine tartrate at a concentration of 33.6 mg/ml and 216 mg/ml for rivastigmine base.

2.2.17. *In vitro* Release Study

Using a 25 G syringe, 0.5 mL of the formulation being tested was injected in each glass tube and placed in a water bath. Pre-warmed PBS (10 mM, pH 7.4) containing 0.01% w/v sodium azide was then added to each tube as release medium (3 mL per tube). The tubes were capped to prevent evaporation and incubated at 37 °C under constant shaking at 35 rpm. Sample aliquots (1 mL) were taken at selected time points and replaced with 1 mL fresh pre-warmed release medium. Released rivastigmine base was quantified using RP-HPLC. A Thermo Scientific C18 column (250 x 4.6 mm, 5 um) and a heated mobile phase of potassium phosphate and methanol was used. Cumulative percent released was calculated over the course of delivery.

2.2.18. *In vivo* Phase Sensitive Formulation Preparation and Experimental Set Up

The *in vivo* procedure was carried out as approved by NDSU IACUC. PLGA (50:50) was dissolved in 95:5 BB:BA at 5% (w/v). Rivastigmine tartrate was suspended via thorough mixing at a concentration of 4.2 mg/ml (0.3 mg/day * 7 days * 2 injections/ml). Conversion from human dose to animal dose was calculated using the following equation: **AED (mg / kg) = Human dose (mg / kg) × K_m ratio**, where Human dose is 0.2 mg/kg (1.2 mg/ 60 kg) and the K_m ratio for a rat is 6.2, giving an AED (animal equivalent dose) of 1.24 mg/kg. Average rat body weight was 0.25 kg which gives a daily dose of 0.3 mg.[158] Rivastigmine tartrate solution was prepared at 0.6 mg/ml in PBS (0.3 mg/day * 2 injections/ml). Animals were divided into groups of 6 for each experimental condition: healthy control (PBS injection), rivastigmine solution and phase sensitive formulation at 4.2 mg/ml rivastigmine tartrate to study release profile and biocompatibility. Three additional groups of 6 animals were used to further study drug concentration at certain time points (15 minutes, 3 days, and 7 days post administration) during the phase sensitive release of rivastigmine tartrate in an effort to investigate effects of the drug on acetylcholinesterase inhibition.

2.2.19. *In vivo* Release Profile of Rivastigmine Tartrate from Phase Sensitive Formulation

Blood samples were drawn from the tail vein of the rats and rivastigmine tartrate was extracted using acetonitrile, table 4. Acetonitrile was added to serum samples and vigorously mixed followed by centrifugation at 4°C. Extracted drug was quantified using RP-HPLC as described in the release study section. Release profile was plotted in the form of drug amount versus time. Time points for sampling are outlined in table 4.

Table 4. Blood Sampling Time Points (n = 6).

Sample Group	Sample Time Post Administration								
	0.25 hr	0.5 hr	1 hr	3 hr	1 day	3 days	7 days	10 days	14 days
Healthy Control	x						x		x
RT Solution	x	x	x	x	x				
Phase Sensitive Formulation	x	x	x	x	x	x	x	x	x

2.2.20. Evaluation of Acetylcholinesterase Inhibition

Acetylcholinesterase inhibition was evaluated post mortem using the Ellman method.[159] A standard curve of acetylcholine concentration was plotted using a serial dilution of acetylthiocholine iodide. Brain samples were homogenized at specific timepoints, table 5. The homogenized brain samples were tested via BCA as a means to normalize all samples. Upon addition of Ellman's reagent, DTNB (5,5-dithio-bis-(2-nitrobenzoic acid), to homogenized samples, DTNB reacts quantitatively with free sulfhydryl groups (—SH) to yield detectable TNB product via calorimetric detection at 412 nm. Therefore, it can quantify acetylcholine which should be at higher concentrations if acetylcholinesterase is inhibited.

Table 5. Time Points for Analysis of Acetylcholinesterase Inhibition in the Brain (n = 6).

Sample Group	Time of Sacrifice				
	15 minutes	1 day	3 days	7 days	14 days
Healthy Control					x
RT Solution		x			
Phase Sensitive Formulation	x		x	x	x

2.2.21. *In vivo* Biocompatibility

Upon animal sacrifice, biocompatibility was evaluated at the injection site. Injection site was dissected and evaluated for signs of inflammation such as vasodilation, redness, and swelling.

2.2.22. Statistical Analysis

All data are expressed as mean ± standard deviation (SD). Statistical analyses were performed using two tailed Student's t-test and ANOVA. A p value of less than 0.05 was considered to be significant.

3. RESULTS

3.1. Synthesis and Characterization of Thermosensitive Triblock Copolymers

Thermosensitive triblock copolymer mPEG-PLGA-mPEG with lactide to glycolide ratio 3.5:1, 4.5:1 and 5:1 were synthesized using ring opening polymerization followed by diblock condensation. The sol to gel transition temperature of the copolymers was tested using tube inversion method. This method allows the determination of temperature at which the copolymer undergoes complete transition from solution to gel form. Copolymers that transition at or below body temperature (37 °C) but remain a solution at room temperature (~27 °C), are physiologically relevant for *in situ* depot formation. In this study, the transition temperatures were observed to be greater than 40 °C for co-polymers with LA:GA, 3.5:1 and 4.5:1. However, copolymer mPEG-PL_5GA_1-mPEG (5:1, LA:GA) transitioned to gel form at 36 ºC and was selected for further characterization. Thereafter, to confirm quick transition from solution to gel, a fresh sample of copolymer was exposed for 30 seconds to physiological temperature. As illustrated in Figure 4, mPEG-PL_5GA_1-mPEG copolymer (LA:GA, 5:1) at different aqueous concentrations (30 and 40% w/v) transitioned successfully from sol to gel in 30 seconds at 37 ºC.

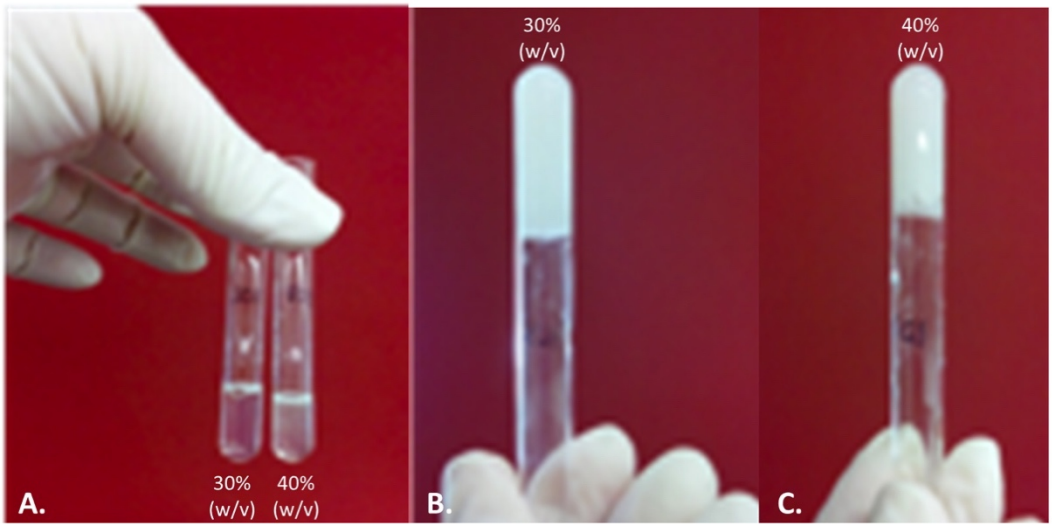

Figure 4. Solution to Gel Transition of mPEG-PLGA-mPEG (LA:GA, 5:1) Copolymer at 30 and 40% (w/v).
Note: Solution to gel transition of mPEG-PL_5GA_1-mPEG (LA:GA, 5:1) copolymer at 30 and 40% (w/v) concentration in DI water at (A) solution form at room temperature (~25 °C), and transition to gel depot (B) 30% (w/v) and (C) 40% (w/v), after incubation at 37 °C for 30 seconds.

3.2. ^{1}H-NMR Spectroscopy

^{1}H-NMR spectra of mPEG-PL_5GA_1-mPEG copolymer confirmed synthesis of mPEG-PLGA-mPEG triblock copolymer with desired lactide to glycolide ratios corresponding to integrals of peaks. ^{1}H-NMR spectra detected peaks at 1.55, 3.38, 3.65, 4.3, 4.8, and 5.2 ppm corresponding to the CH_3 of LA, CH_3 of mPEG end group, CH_2 of mPEG, CH_2 between PLGA and mPEG, CH_2 of GA, and CH of LA, respectively (Figure 5).

3.3. Gel Permeation Chromatography

Molecular weight distribution of the mPEG-PL_5GA_1-mPEG (LA:GA, 5:1) copolymer was determined using GPC size determination and to provide evidence of the homogeneity of the copolymer (polydispersity index). Number average molecular weight (Mn) and weight average molecular weight (Mw) of the copolymer were found to be 2,950 and 4,180 Da, respectively while PDI was found to be ~1.42 (Figure 6).

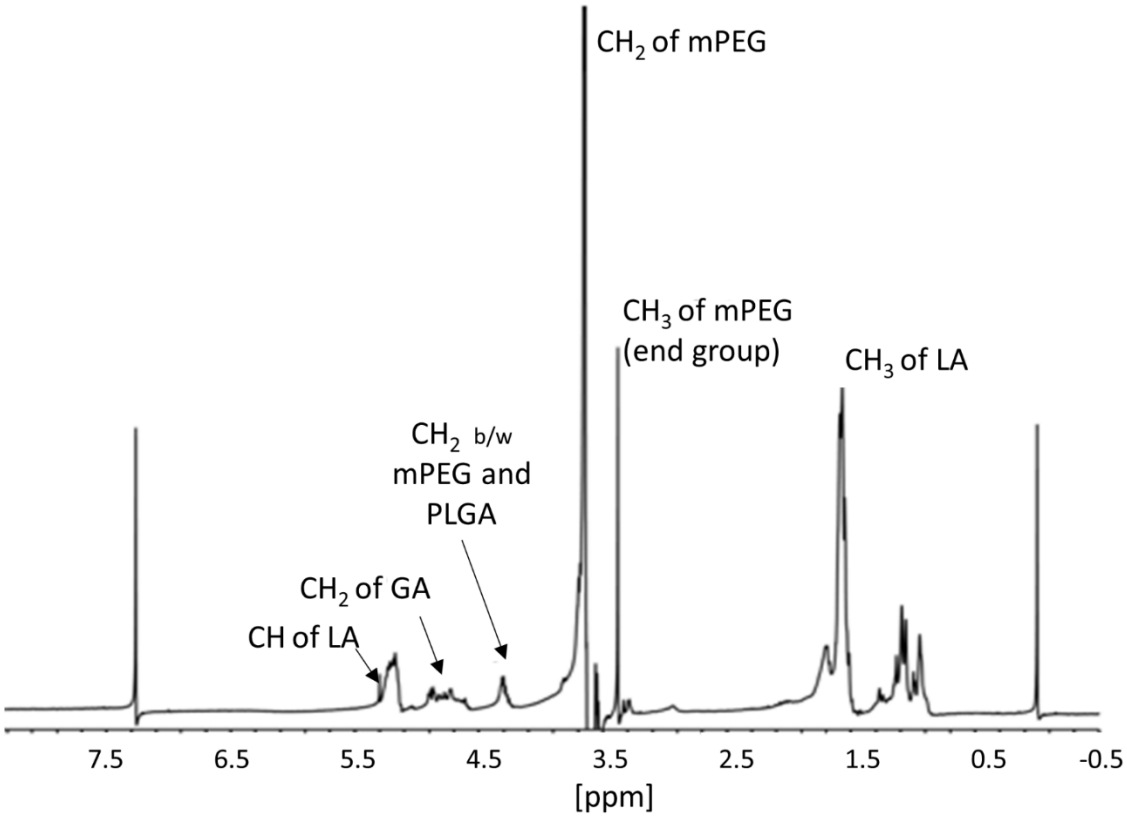

Figure 5. ^{1}H NMR Spectra of mPEG-PLGA-mPEG (LA:GA, 5:1) Copolymer.

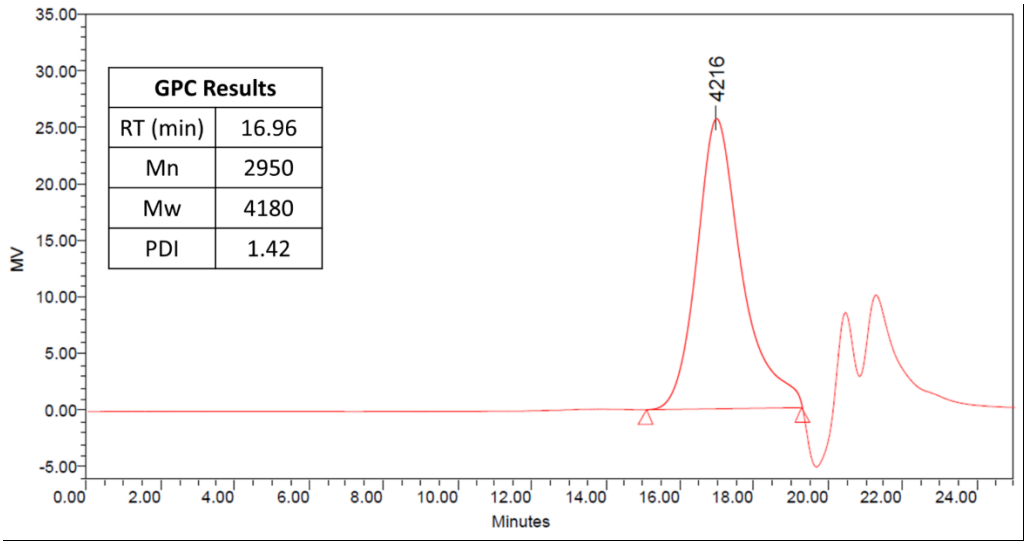

Figure 6. GPC Chromatogram of mPEG-PLGA-mPEG (LA:GA, 5:1) Copolymer.

3.4. Critical Micelle Concentration

CMC of mPEG-PL_5GA_1-mPEG (LA:GA, 5:1) copolymer was determined using pyrene fluorescence probe method and was found to be 25 µg/mL (Figure 7). Transition from solution to gel is based upon hydrophobic effect and ability of the polymer to arrange itself into an ordered micellar structure. Low CMC value supports the amphiphilic structure of the copolymer that organizes into fairly stable micelles at relatively low concentration.

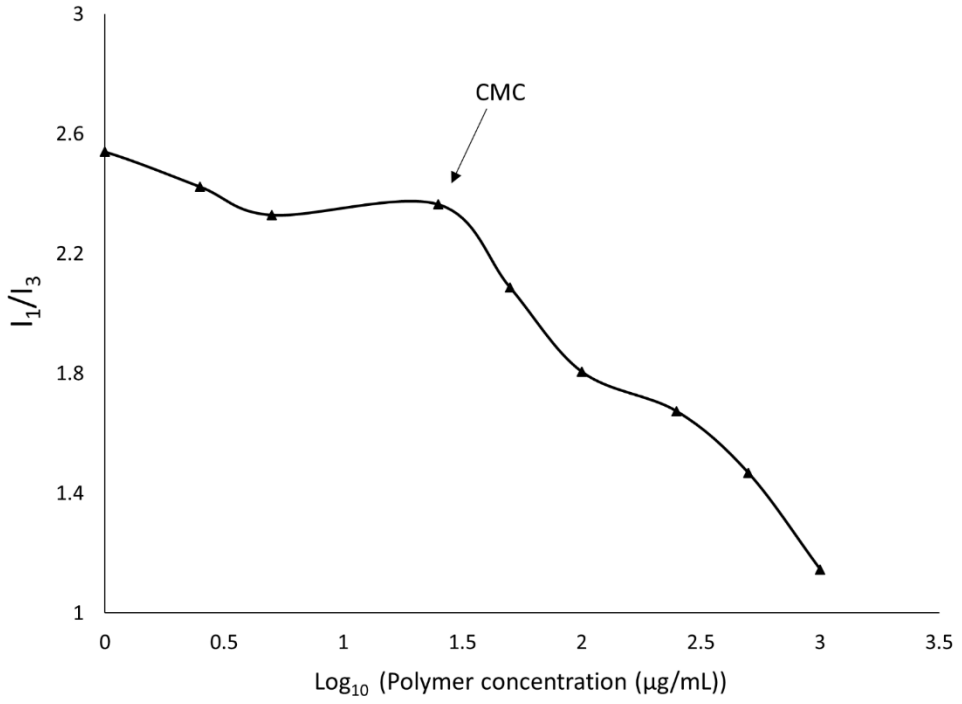

Figure 7. Critical Micelle Concentration of mPEG-PLGA-mPEG Using Pyrene as a Fluorescent Probe.
Note: Plot of pyrene fluorescence intensity ratio (I_1/I_3) versus decadic logarithm of copolymer concentrations in deionized water at room temperature for mPEG-PL_5GA_1-mPEG (LA:GA, 5:1) copolymer. Arrow indicates the critical micelle concentration of the copolymer.

3.5. *In vitro* Biocompatibility Assay

In vitro biocompatibility of mPEG-PL_5GA_1-mPEG (LA:GA, 5:1) copolymer was evaluated by testing different concentrations of the copolymer in HEK 293 cells by MTT cell viability assay. Biocompatibility in HEK 293 is widely considered necessary to support cyto-compatibility *in vivo* and assess adverse reactions of the sample. Compared to control, the viability of cells incubated with copolymer samples was found to be higher than 80% when tested up to a concentration of 1 mg/mL (Figure 8). However, increasing copolymer concentration beyond 1 mg/mL reduced cell viability. The IC_{50} of the copolymer was found to be >10 mg/ml, which suggests high cyto-compatibility of the copolymer.

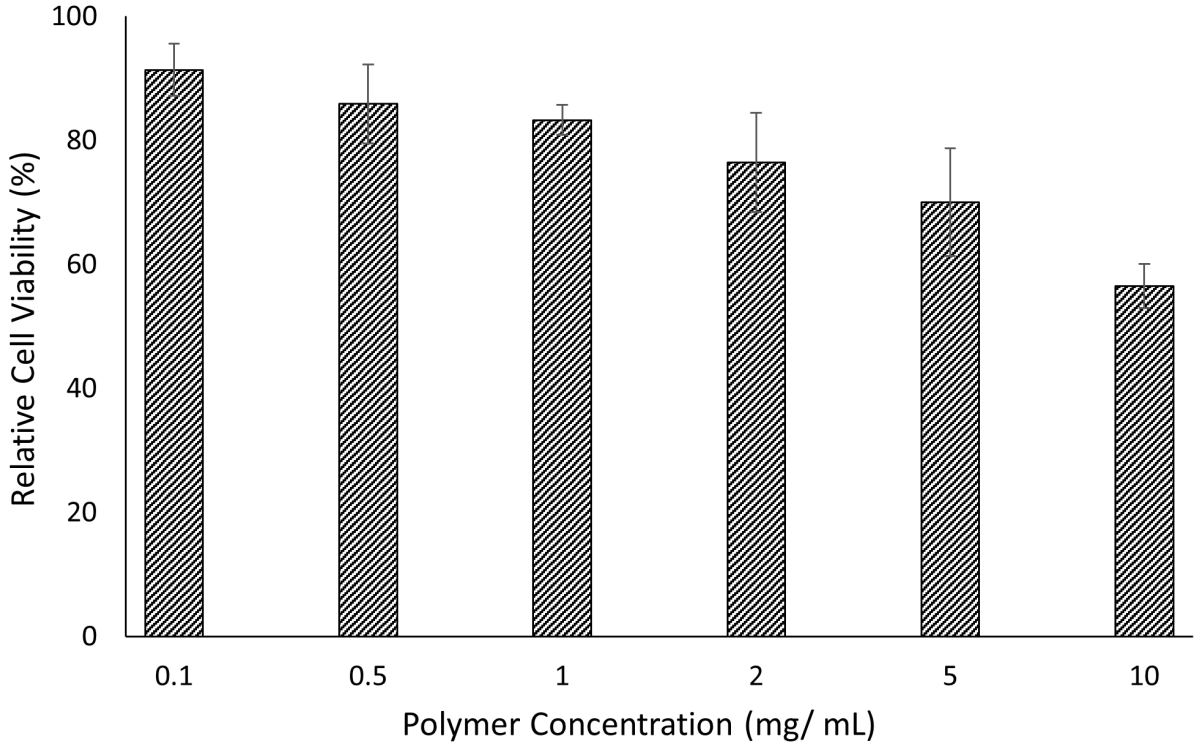

Figure 8. *In vitro* Cell Viability of mPEG-PLGA-mPEG.
Note: Graphical representation of percent relative cell viability at different concentrations of mPEG-PL_5GA_1-mPEG (LA:GA, 5:1) copolymer. Data represent the mean ± SD (n = 4).

3.6. *In vitro* Release Profile of Salmon Calcitonin

Formulations composed of sCT incorporated in mPEG-PL_5GA_1-mPEG (LA:GA, 5:1) thermosensitive triblock copolymer (30 and 40% w/v) were easily injectable using a 25 G syringe and transitioned instantaneously into a gel upon incubation at 37 ˚C. Percent cumulative release of sCT from thermosensitive copolymer formulations were studied *in vitro*. In order to be most effective, the release of therapeutics from a controlled release delivery system should follow zero order kinetics so that a constant level of the drug is maintained continuously in circulation and hence produce sustained action. *In vitro* release profile mimics what can be expected to be seen *in vivo* which allows for optimization needed prior to *in vivo* studies. Factors such as burst release, release rate, and complete release are important in optimization of a sustained release formulation based on the drug's therapeutic index, toxicity profile and mode of action. Looking at two concentrations of copolymer formulations allows us to optimize the formulation to achieve the best release profile. Higher concentrations of copolymer within the formulation will result in slower release over time due to a few factors such as slower degradation rate and increased viscosity through which therapeutics would need to overcome in order for realease from diffusion to occur. An initial burst release of sCT at 10.6 ± 0.58 % and 7.7 ± 3.8 % from the 30 and 40% formualtions, respectively, was observed. Burst relase was followed by a steady release for up to 49 and 70 days, respectively for the two formulations and amounted to a cumulative sCT release of 103.2 ± 4.64 % and 106.4 ± 6.16 % for the 30 % and 40% formulations (Figure 9). A low burst, such as that shown in this system, is desirable to avoid toxicity due to high drug concentrations in the body. The study also demonstrates the stability of the depot and provides insights into its ability to control the release of incorporated therapeutic.

The release profile correlation coefficients (r^2 values) best fit zero order release with values of 0.991 and 0.942, respectively for 30% and 40% formulations.

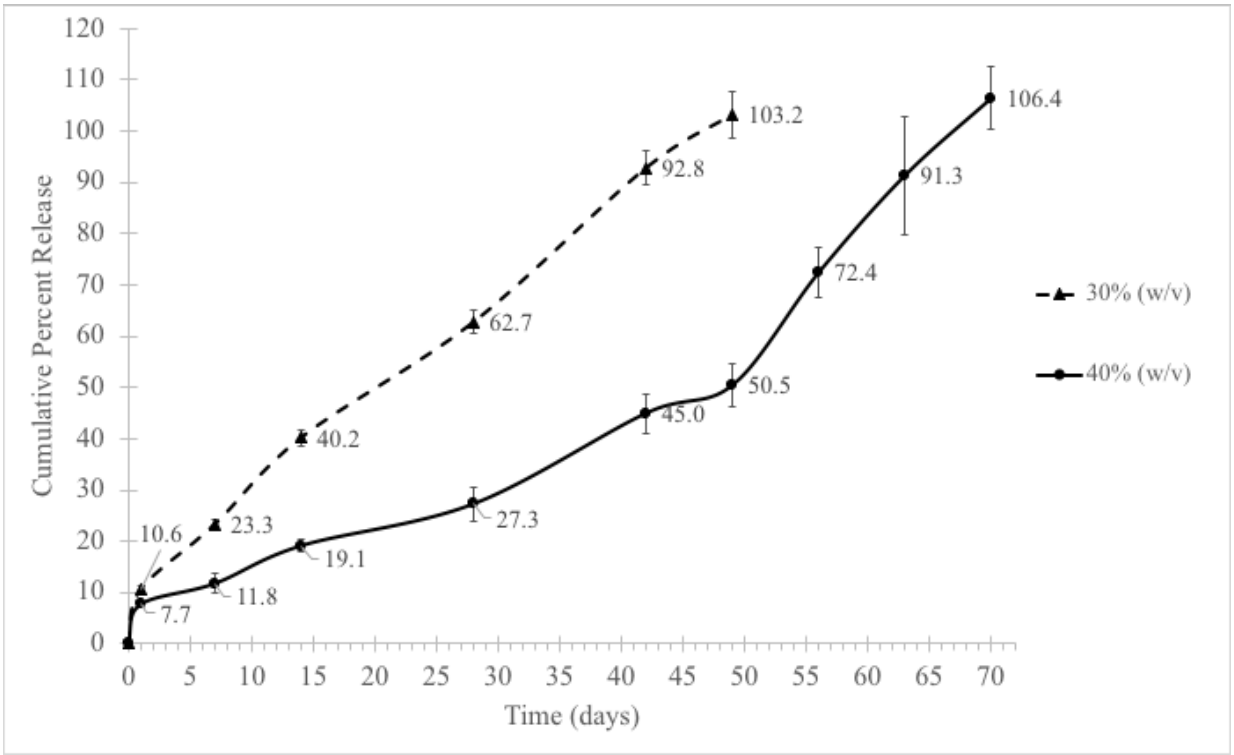

Figure 9. *In vitro* Cumulative Percent Release of Salmon Calcitonin from mPEG-PLGA-mPEG (LA:GA, 5:1).
Note: Percent cumulative release of salmon calcitonin *in vitro* from mPEG-PL_5GA_1-mPEG (LA:GA, 5:1) thermosensitive triblock copolymer (30 and 40% w/v). Data represent the mean ± SD (n = 4).

3.7. Stability of Salmon Calcitonin

Conformational changes in sCT were evaluated both at physiological and storage temperatures of 37 ˚C and 4 ˚C. Stability of salmon calcitonin was confirmed

3.7.1. Stability of Salmon Calcitonin at Physiological Temperature

Released sCT must be in its native conformation in order to interact with its receptor and demonstrate bioactivity. CD spectra of sCT released from copolymer depot at 37 °C withdrawn at 15 and 30 days, showed characteristic minima at ~205 nm, corresponding to the freshly

prepared sCT (Figure 10 A). Secondary structure analysis of freshly prepared sCT and sCT released from copolymeric formulation is reported in Table 6 A. This analysis of sCT released from the copolymer supports its protection from denaturation inside the polymer depot and retention of its bioactive conformation upon release.

3.7.2. Stability of Salmon Calcitonin Inside the Gel During Storage at 4 °C

Preparation and storage of therapeutics play a large role in the assessment of its suitability for future development and use in the clinics. Stability of sCT incorporated in gel depot was assessed to make sure that the structure of the protein remains unaltered during storage at 4 °C. CD spectra of sCT extracted from copolymer depot stored at 4 °C, at 15 and 30 days, showed minima at 208 and 222 nm, which is typical of an alpha-helix structure and characteristic of native conformational structure of sCT in the presence of organic solvent, as compared to the standard solution comprising freshly prepared sCT in PBS:ACN, 1:1 v/v (Figure 10 B). Secondary structure analysis of standard and sCT extracted from stored copolymer depots is reported in Table 6 B.

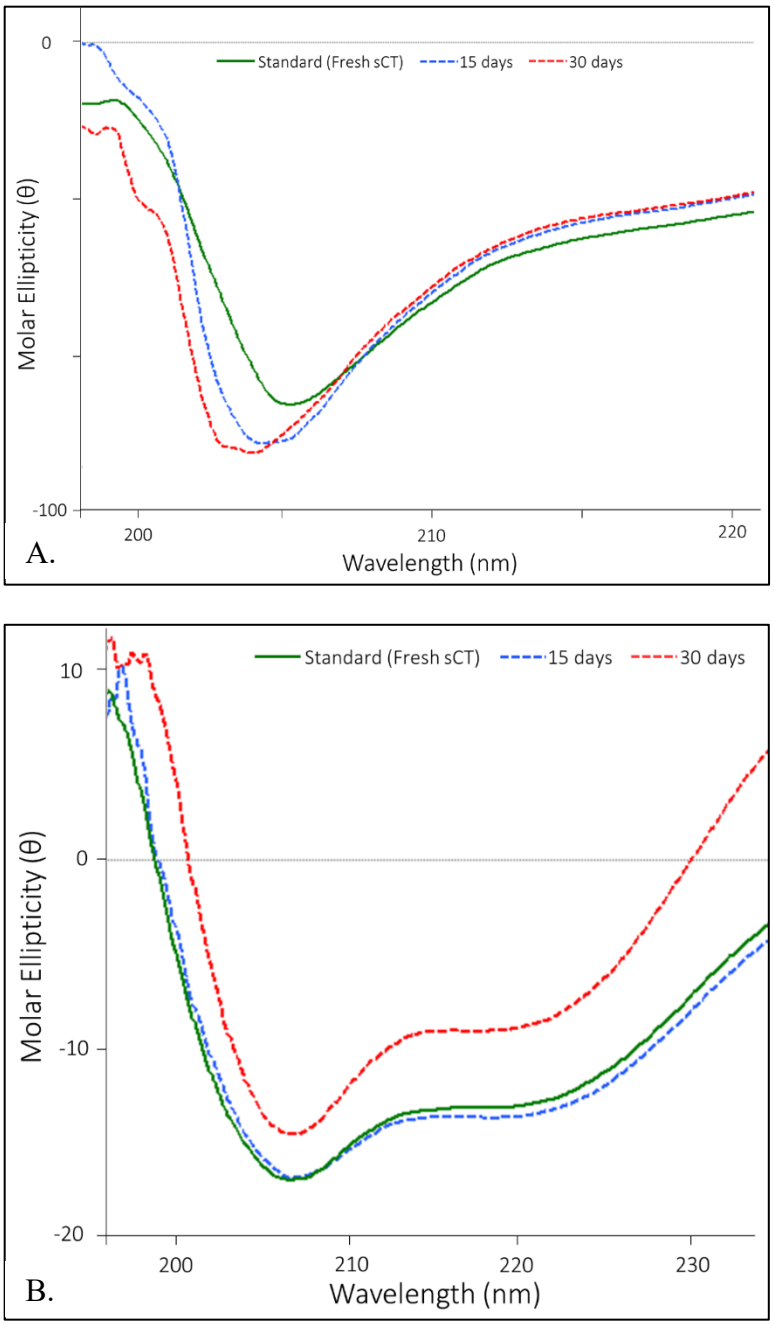

Figure 10. Circular Dichroism Spectra of Stable Salmon Calcitonin Under A) Release Conditions and B) Storage Conditions.

Note: A) Circular dichroism spectra depicting stability of sCT released from polymeric formulations. The spectra shows native sCT (freshly prepared solution) and sCT released from the polymeric formulations at 37 ˚C, withdawn at days 15 and 30. (n=4) B) Circular Dichroism spectra depicting storage stability of sCT. The spectra show native sCT (freshly prepared solution) and sCT extracted from the polymeric formulation after 15 and 30 days, stored at 4 ˚C. (n=4)

Table 6. Secondary Structure Analysis of Salmon Calcitonin

A.

	Sample	Days	α Helix	β Sheets	β Turns	Random Coils
Salmon calcitonin released from copolymeric formulations in release buffer at 37˚C	Fresh sCT in PBS	-	9.5	46.9	11.7	31.9
	sCT released in PBS	15	7.1	54.2	9.3	29.5
	sCT released in PBS	30	3.9	64.2	0	31.8

B.

	Sample	Days	α Helix	β Sheets	β Turns	Random Coils
Salmon calcitonin extracted from copolymeric formulations stored at 4˚C	Fresh sCT in PBS:ACN (1:1)	-	16.2	35.6	3.9	44.3
	sCT extracted from copolymer depot	15	14.9	40.7	1	43.5
	sCT extracted from copolymer depot	30	13.4	41.6	1.7	43.4

Note: A) Secondary structure analysis of salmon calcitonin released from polymeric formulations in release buffer at 37 ˚C and B) extracted from polymeric formulations stored at 4˚C.

3.8. *In vitro* Release Profile Comparing Polymer Concentration of Thermosensitive Formulations

Polymer concentration effected rivastigmine tartrate release profile in a couple of ways. At a concentration of 20, 30, and 35% (w/v) a burst release of 36, 32, and 32% was observed, respectively and release was maintained until day 7 for all polymer concentrations tested, figure

11. Burst release can have major consequences and any reduction is a better controlled release formulation. The release profiles for 30 and 35% (w/v) show an almost biphasic release with the 35% release profile being most noteworthy. Although biphasic release is not unusual for controlled release systems, is not desirable. Since the goal of controlled release is to maintain a therapeutic drug level over the entire course of release, a plateau in which drug release slows can have negative effects on symptom relief and effectiveness of the medication.

On the other hand, polymer concentration did not appear to have much effect on release profile for rivastigmine base until reaching a concentration of 35% (w/v), figure 12. At concentrations of 20, 25, and 30%, rivastigmine base release was maintained until day 9 with the 30% (w/v) concentration outperforming the others in terms of controlling burst release and providing a more even nature of release. However, the 35% (w/v) formulation demonstrated zero order kinetics over the course of 14 days while minimizing burst release. Burst release is an important factor to investigate because if too high, it can cause significant toxicity. Burst release can also influence the overall release profile in a number of ways such as decreasing the length of release and opening up more pores and channels to allow for faster drug diffusion and degradation of polymer. Based on the superior release profile obtained using 35% w/v polymer, this concentration was utilized for our further studies using thermosensitive formulations.

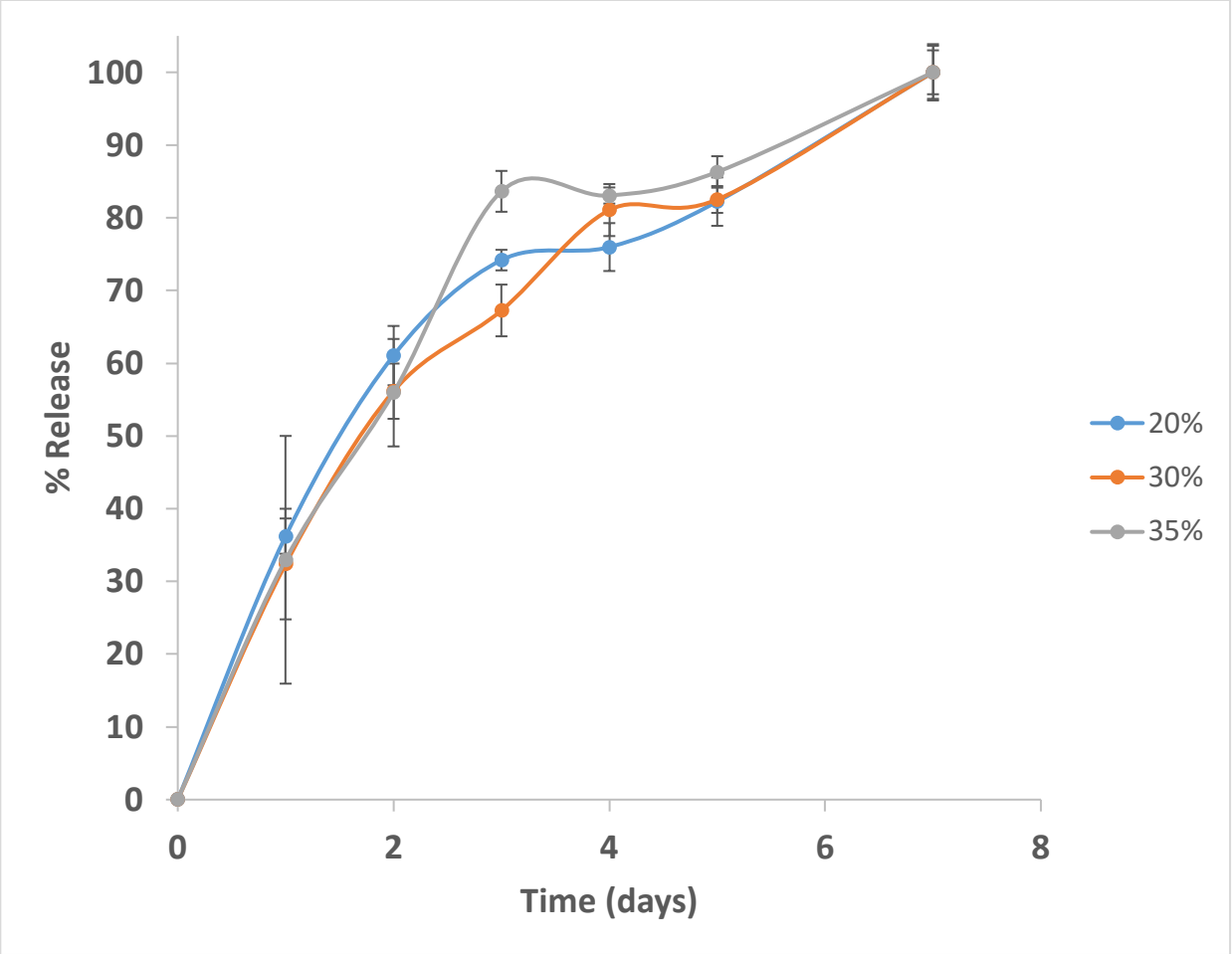

Figure 11. *In vitro* Release Profiles of Rivastigmine Tartrate Comparing Copolymer Concentration (n=6).

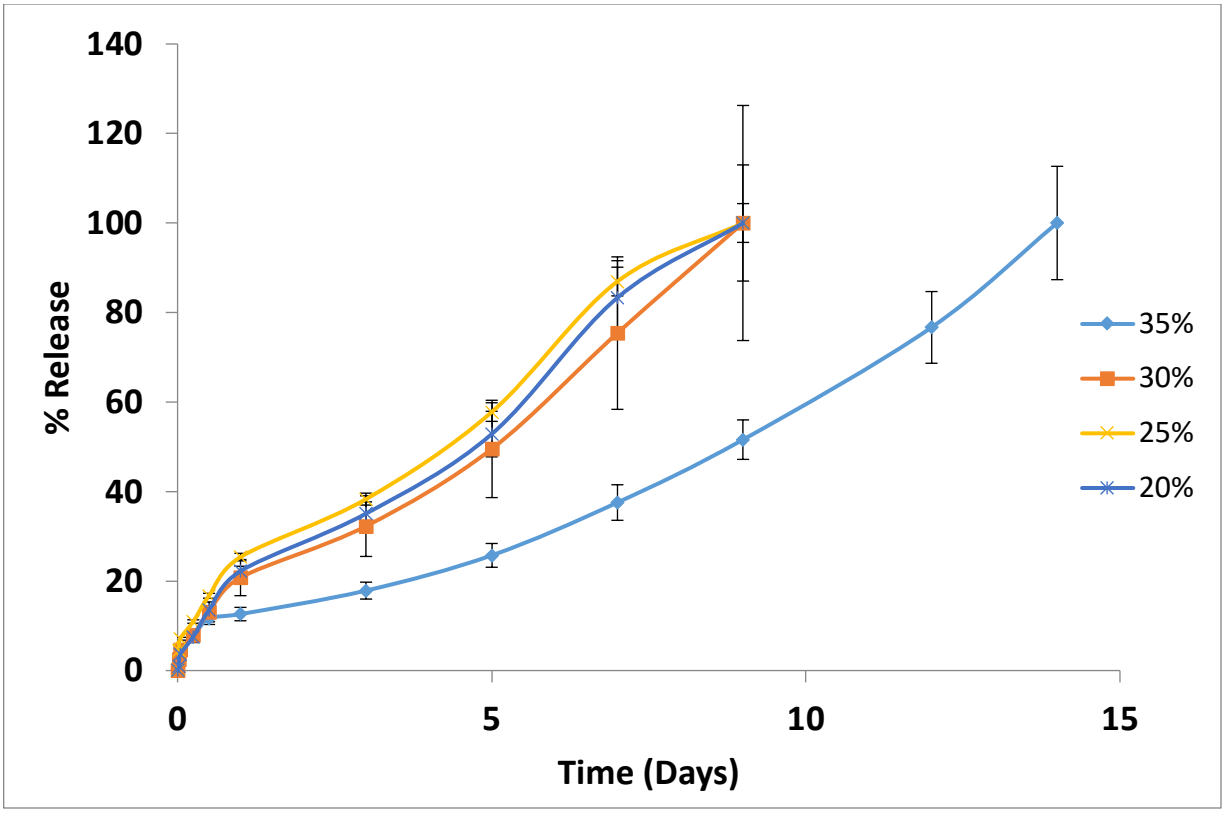

Figure 12. *In vitro* Release Profiles of Rivastigmine Base Comparing Copolymer Concentration (n=6).

3.9. *In vitro* Release Profile Comparing Therapeutic Concentration in Thermosensitive Formulations

The concentration of rivastigmine tartrate incorporated in 1 ml of 35% (w/v) copolymer formulation had a couple of impacts on release profile. First, the burst release decreased as concentration of rivastigmine tartrate increased. However, since the actual amount of drug released in 24 hrs is higher as the concentration increases, the percent release does not provide a complete picture of drug release. Secondly, increasing the concentration of rivastigmine tartrate, increased the duration of release, but not proportionately. When 5, 18, and 36 mg/ml was incorporated in the formulation, the duration of release was 7, 18, and 20 days, respectively, figure 13. Similarly, for rivastigmine base, the duration of release for 5, 10, 20, and 40 mg/ml was 12, 14, 13, and 16 days, respectively (figure 14). The length of release can be an important

factor when optimizing the amount of drug to be incorporated in the formulation in order to maintain therapeutic levels within the body. It is also very important when toxicity has severe consequences, or the therapeutic window is narrow. For rivastigmine, the daily doses of 3-12 mg/day equates to a distribution of 1-400 ng/ml. Concentrations above this window can result is severe adverse reactions such as severe nausea, vomiting, salivation, sweating, bradycardia, hypotension, respiratory depression, collapse, convulsions, or even death.

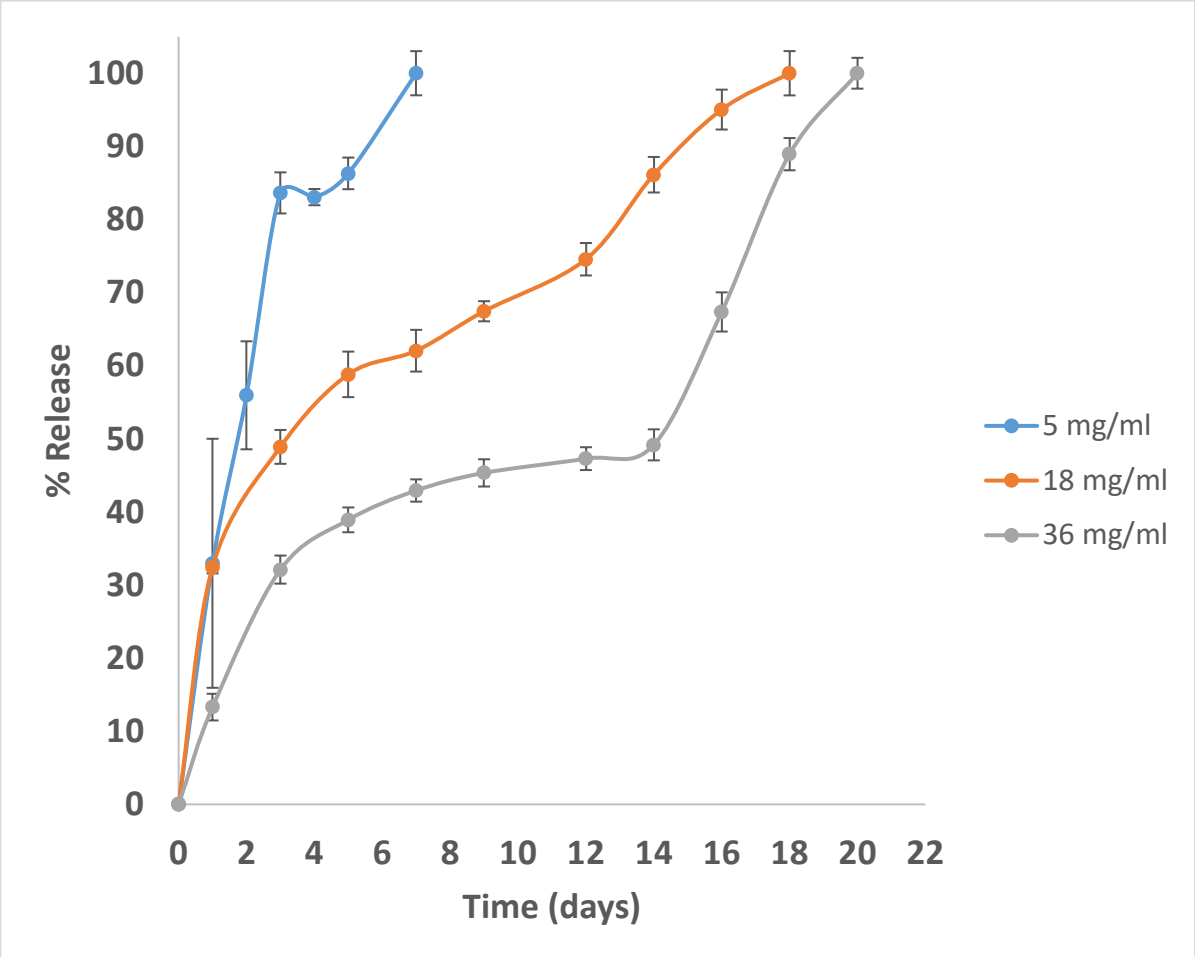

Figure 13. *In vitro* Release Profiles of Rivastigmine Tartrate Comparing Therapeutic Concentration (n=6).

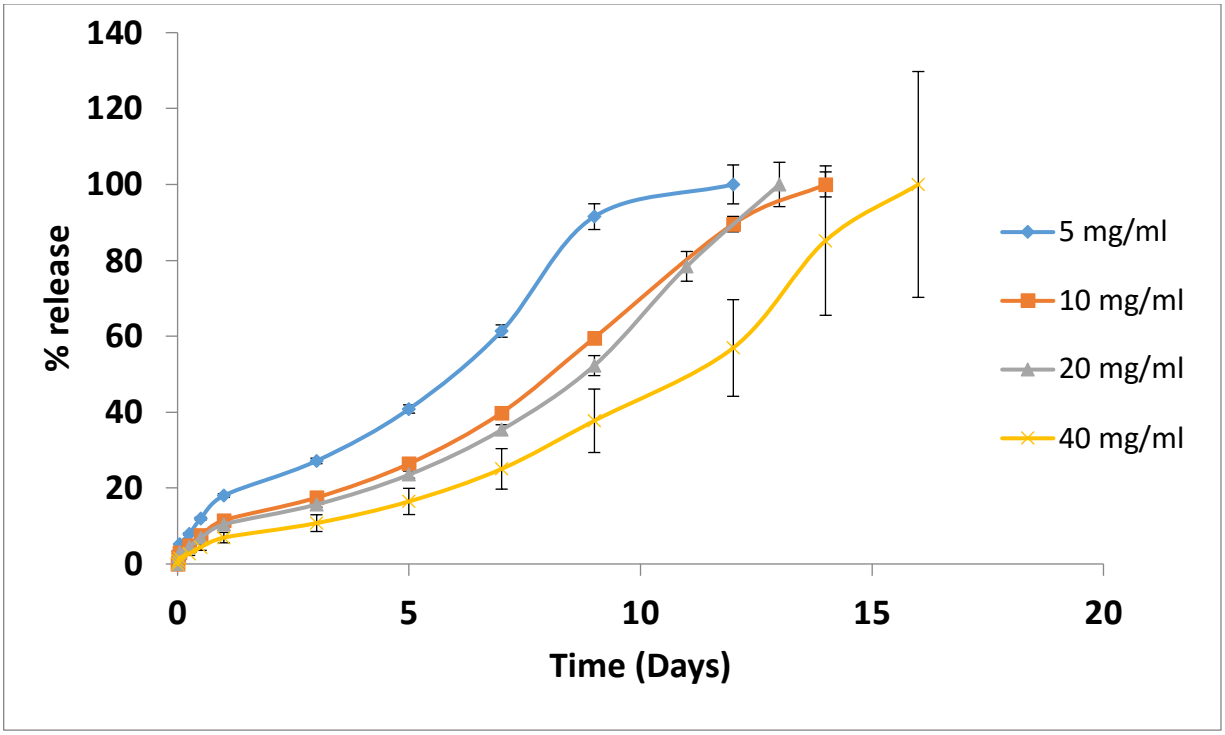

Figure 14. *In vitro* Release Profiles of Rivastigmine Base Comparing Therapeutic Concentration (n=6).

3.10. *In vitro* Release Profile Comparing Depot Volume of Thermosensitive Formulations

Depot volume is an important parameter to investigate because it could influence release, but moreover, it can be a limiting factor to optimization. Thermosensitive smart polymers are designed to be injected subcutaneously. The space in which the formulation can be injected, and depot formed is limited and usually kept to around 1.5 ml or less. The smaller the injection volume, the better tolerated the injection will be. Burst release is largely contributed to the therapeutic that is closest to the surface of the depot diffusing easily into surrounding interstitial fluid. In this study, there was no significant difference in drug release compared to depot volume. The rivastigmine base release duration of the 0.5, 1.0, and 1.5 ml depot volumes was 14, 13, and 14 days, respectively (figure 15). The 0.5 ml depot volume showed highest burst release in terms of cumulative percent release, but the drug content in the formulation was also lower than the other two formulations. Order of release is something that can be impacted as seen in this

comparison and should therefore be considered when optimizing release profile since the goal is to maintain a constant level within the body by releasing a constant amount of drug.

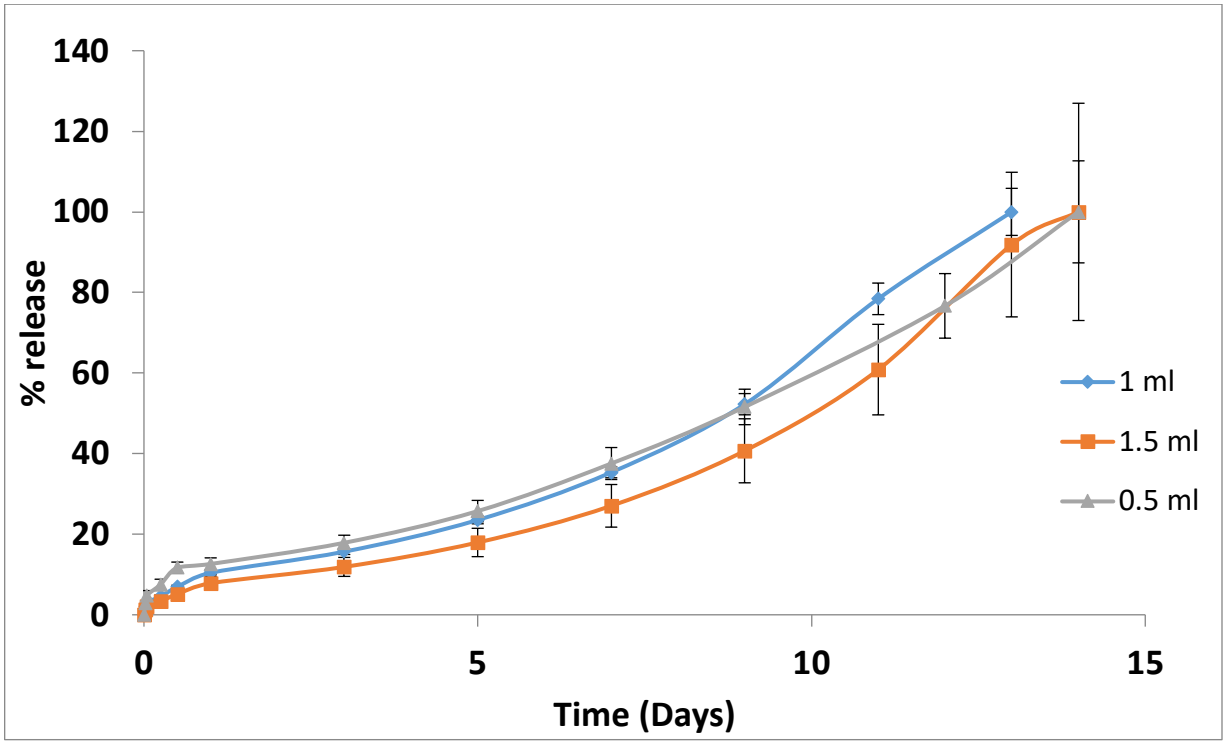

Figure 15. *In vitro* Release Profiles of Rivastigmine Base Comparing Depot Volume (n=6).

3.11. *In vitro* Release Profile Comparing Therapeutic Hydrophobicity in Thermosensitive Formulations

By comparing the release profiles of rivastigmine tartrate and rivastigmine base, we can look at the influence hydrophobicity has on release rate and order of release, figure 16. The thermosensitive formulation was able to control the release rivastigmine tartrate over the course of 9 days while providing controlled release of rivastigmine base for 12 days. Rivastigmine tartrate is hydrophilic and readily dissolves in water. Incorporation into the micelles that make up the depot should be in the hydrophilic shell. The surrounding aqueous solution will be a driving force for rivastigmine tartrate to diffuse out of the polymer to the more hydrophilic surrounding environment. This driving force is typically responsible for the large burst release that may be

observed with hydrophilic drugs. In addition, it can contribute to the biphasic release that may be observed. In contrast, rivastigmine base is hydrophobic. It will partition to the core of the micelles that form upon solution to gel transition. The reluctance of rivastigmine base to diffuse into the hydrophilic environment due to its hydrophobicity, will slow down the release rate and can even shift the driving force for release of drug from diffusion to polymer breakdown. In this case, release profile tended to follow zero order release kinetics and greatly decreased burst release. Rivastigmine base provided a release profile that was much better controlled over the course of delivery.

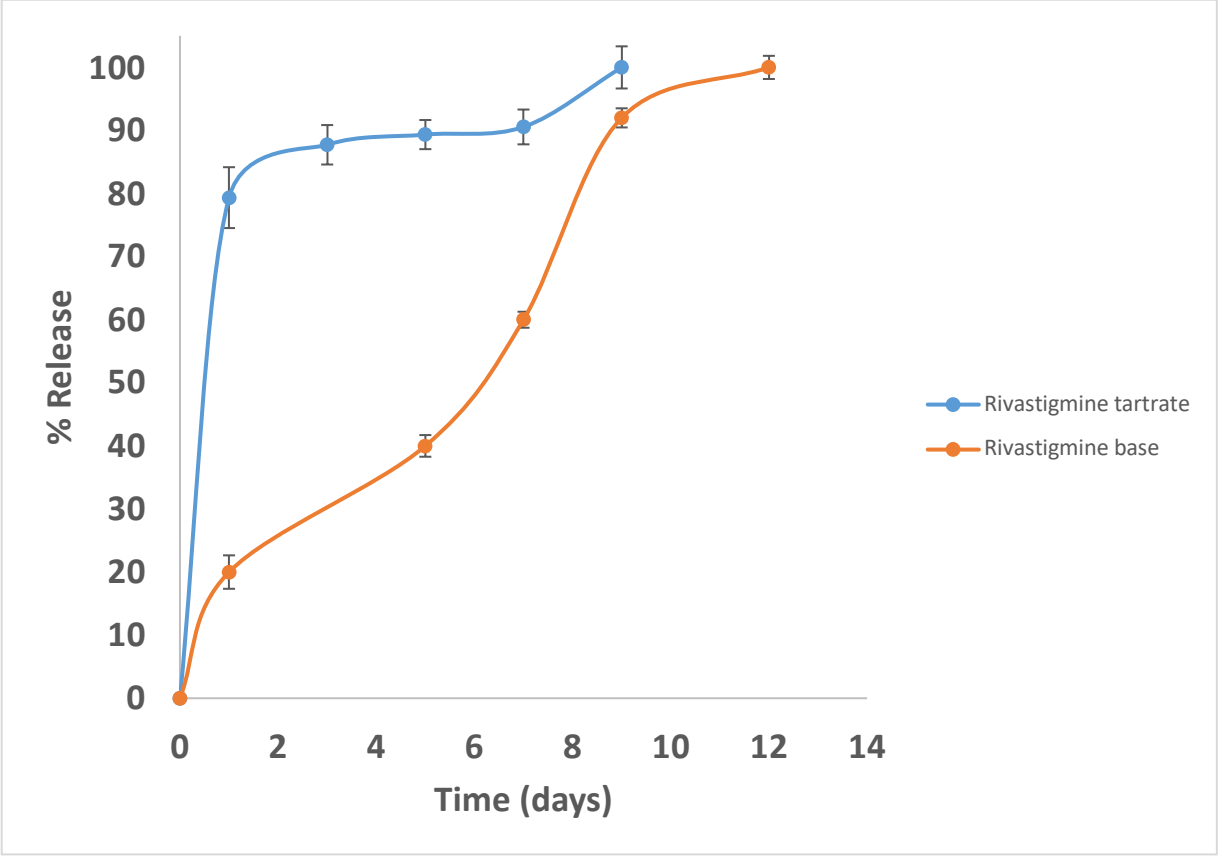

Figure 16. *In vitro* Release Profiles of Rivastigmine Tartrate and Rivastigmine Base Comparing Therapeutic Hydrophobicity (n=6).

3.12. *In vitro* Release Profile Comparing Polymer Concentration of Phase Sensitive Formulations

Formulations containing 60 mg/ml rivastigmine base composed of PLA (109 kDa) copolymer at weight to volume ratios of 2.5, 5, and 10% in 100% benzyl benzoate were injected in each glass tube using a 25 G syringe at a volume of 0.5 mL and release profile was compared, figure 17. Release profiles did not follow an expected trend of increased release duration upon increased polymer concentration. Instead, there is a balance of hydrophobic effect influencing release profile. Hydrophobic effect is driven by reduction in entropy. At certain concentrations of polymer, the monomers of the polymer will be able to better arrange themselves in an effort to reduce entropy as much as possible, forming a more energetically favorable state. This is clearly evident in this study as the best performing concentration of polymer is 5% (w/v) with a release duration of 77 days and minimal burst release. All formulations had commendable control over burst release at 3.5- 6%. The release duration was 42 days for the 2.5 % (w/v) formulation and 49 days for the 10% (w/v) formulation. Based on the superior drug release profile demonstrated by the 5% w/v formulation, it was utilized for further optimization studies.

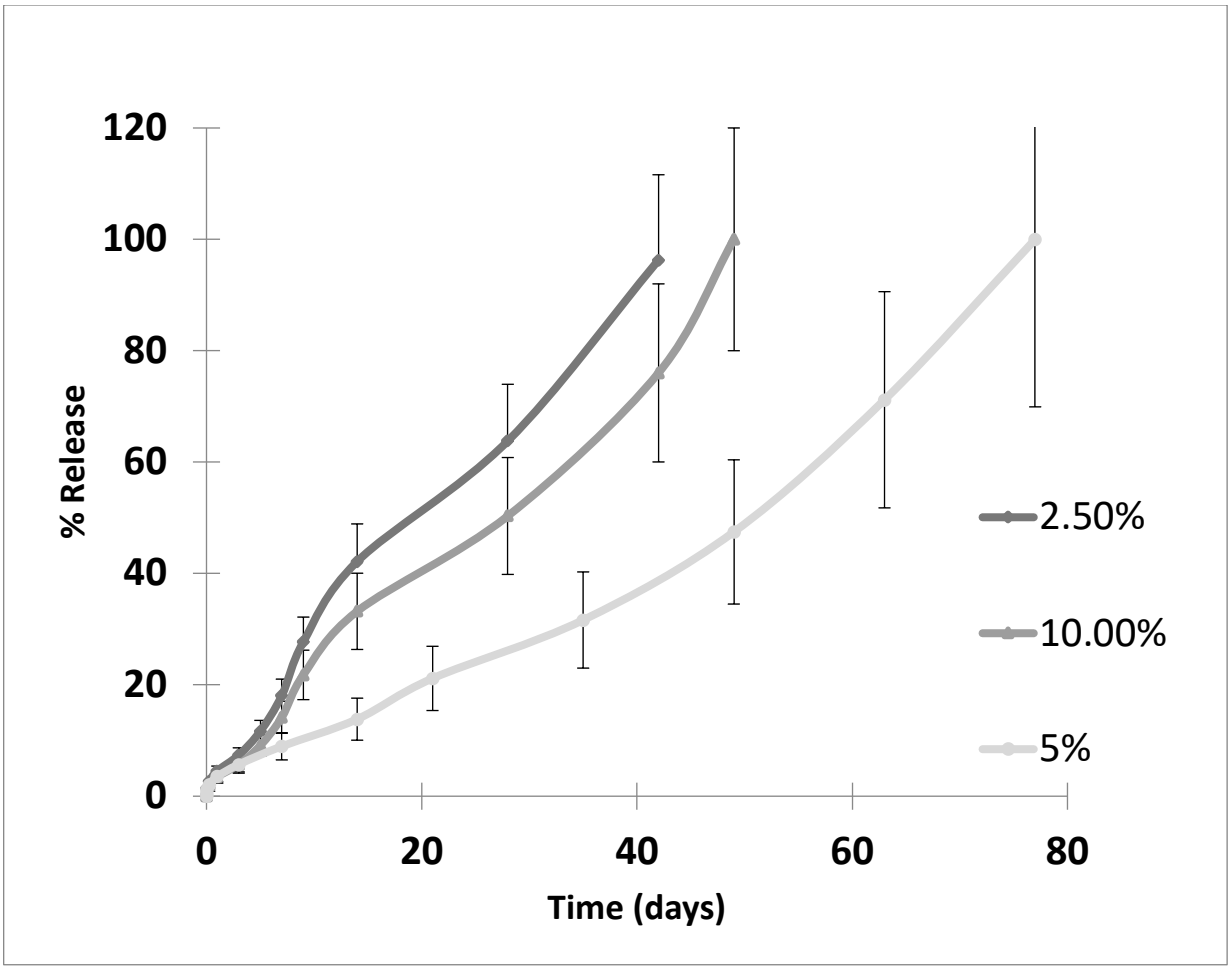

Figure 17. *In vitro* Release Profiles of Rivastigmine Base Comparing Concentrations of PLA (109 kDa) Polymer (n=6).
Note: PLA (109 kDa) was formulated in solvent composed of 100:0 BB:BA.

3.13. *In vitro* Release Profile Comparing Therapeutic Concentration in Phase Sensitive Formulations

Formulations containing 60, 120, and 180 mg/ml rivastigmine base composed of PLA (109 kDa) copolymer at weight to volume ratios of 5% in 100% benzyl benzoate were injected in each glass tube using a 25 G syringe at a volume of 0.5 mL and release profile was compared, figure 18. Release duration was 49, 77, and 98 days for the 60, 120, and 180 mg/ml formulations, respectively. Length of release was not proportional to drug concentration but increase in drug

concentration resulted in increased duration of release. Burst release was minimal in all formulations.

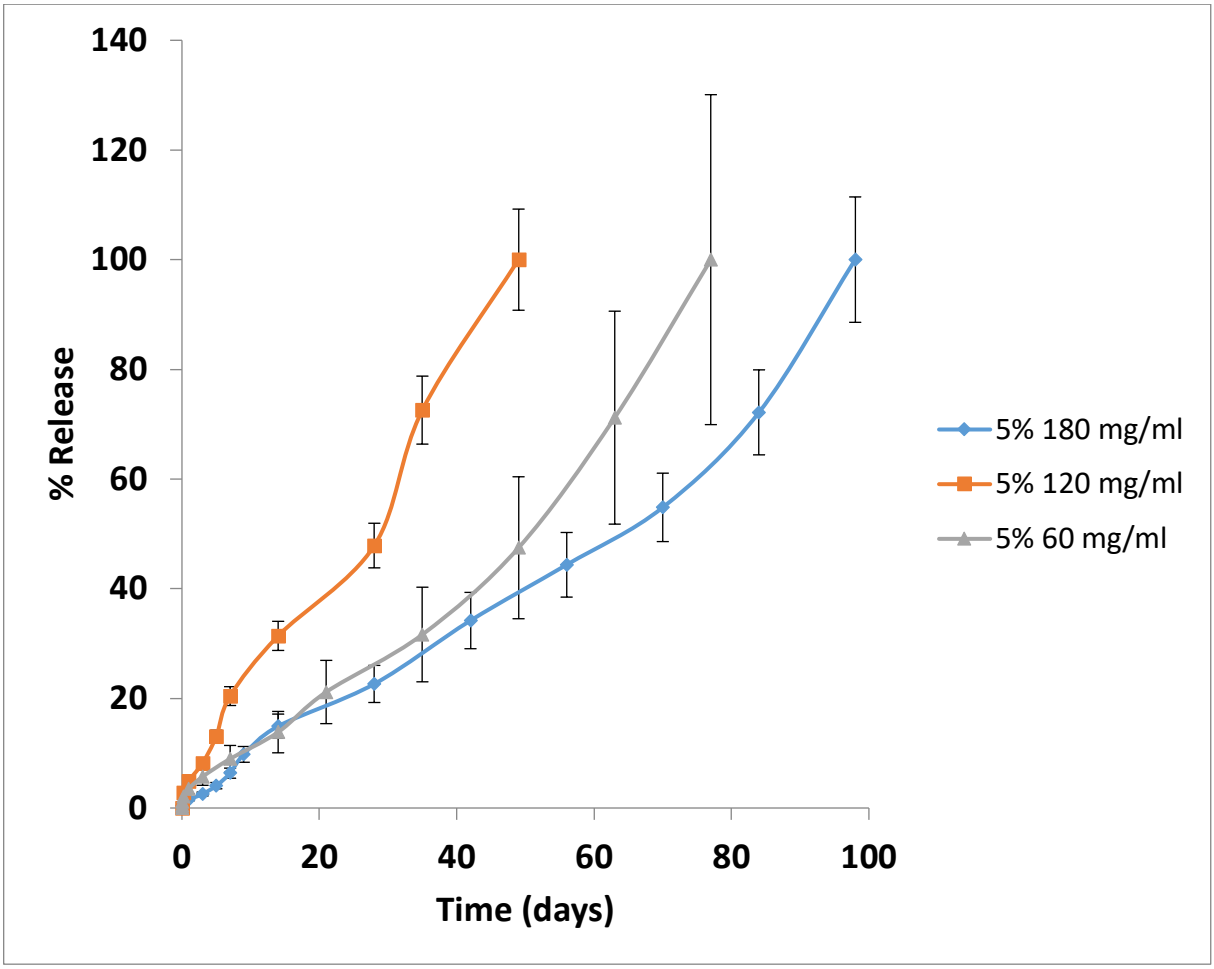

Figure 18. *In vitro* Release Profiles of Rivastigmine Base Comparing Therapeutic Concentration from Formulations of PLA (109 kDa) in BB (n=6).
Note: PLA (109 kDa) was formulated in 100:0 BB:BA.

3.14. *In vitro* Release Profile Comparing Polymer Composition of Phase Sensitive Formulations

Formulations containing 216 mg/ml rivastigmine base composed of PLA (109 kDa), PLA (40 kDa), PLGA (85:15), or PLGA (50:50) copolymer at weight to volume ratios of 5% in 100% benzyl benzoate were injected in each glass tube using a 25 G syringe at a volume of 0.5 mL and release profile was compared, figure 19. Release duration was 35 days for both PLGA

formulations, 42 days for the PLA (40 kDa) formulation, and 49 days for the PLA (109 kDa) formulation. While the duration of release was longer for the PLA formulations, it also followed a more biphasic release pattern when compared to the profiles of PLGA. Burst release was well controlled for all formulations.

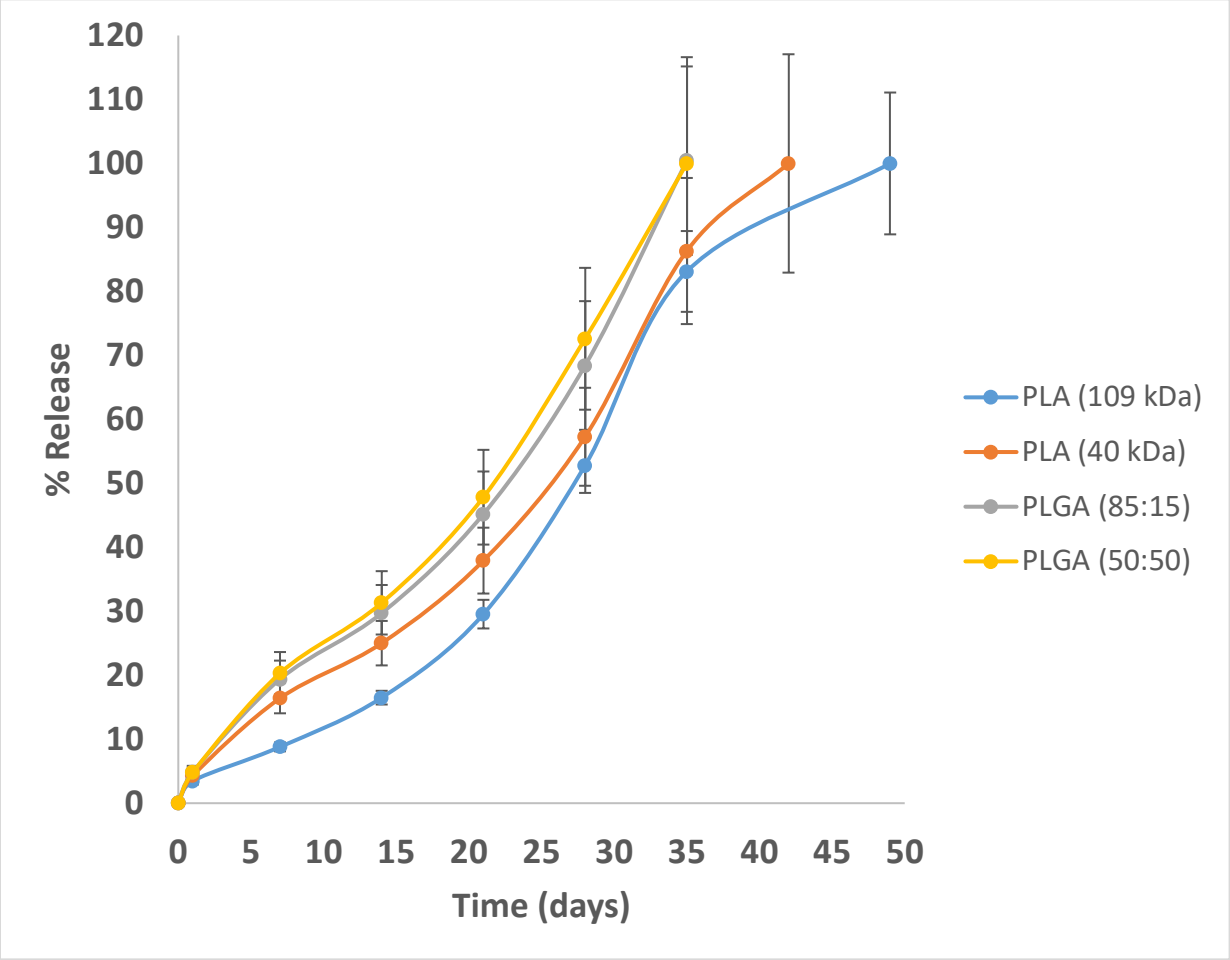

Figure 19. *In vitro* Release Profiles of Rivastigmine Base Comparing Polymer Composition (n=6).
Note: Solvent used for formulations was 100:0 BB:BA.

3.15. *In vitro* Release Profile Comparing Solvent Composition of Phase Sensitive Formulations

Formulations containing 216 mg/ml rivastigmine base composed of PLA (109 kDa), PLA (40 kDa), PLGA (85:15), or PLGA (50:50) copolymer at weight to volume ratios of 5% but varying in solvent compositions of benzyl benzoate to benzyl alcohol ratios (100:0, 95:5, 90:10, 85:15) were injected in each glass tube using a 25 G syringe at a volume of 0.5 mL and release profile was compared, figures 20-23. Release duration was 49 days for PLA 109 kDa at solvent composition of 100:0 BB:BA and 42 for the other solvent compositions. For PLA 40 kDa, release of all solvent compositions was 56 days. As for PLGA 85:15, release of 100:0 and 95:5 BB:BA compositions was 42 days while 90:10 and 85:15 BB:BA had a slightly longer duration of 49 days. Finally, PLGA 50:50 showed release duration of 49 days at all solvent compositions. While the duration of release was the longest at 56 days for the PLA (40 kDa) formulation in this study, it still followed a more biphasic release pattern when compared to the profiles of PLGA. Burst release was well controlled for all formulations but a trend was observed for increased burst release as benzyl alcohol composition increased. It is worthy to note that given the factors of burst release, duration of release, zero order release, amount of drug release per day, and standard deviation among formulation samples, the PLGA (50:50) in 95:5 BB:BA formulation could potentially be best suited for controlled delivery of rivastigmine base.

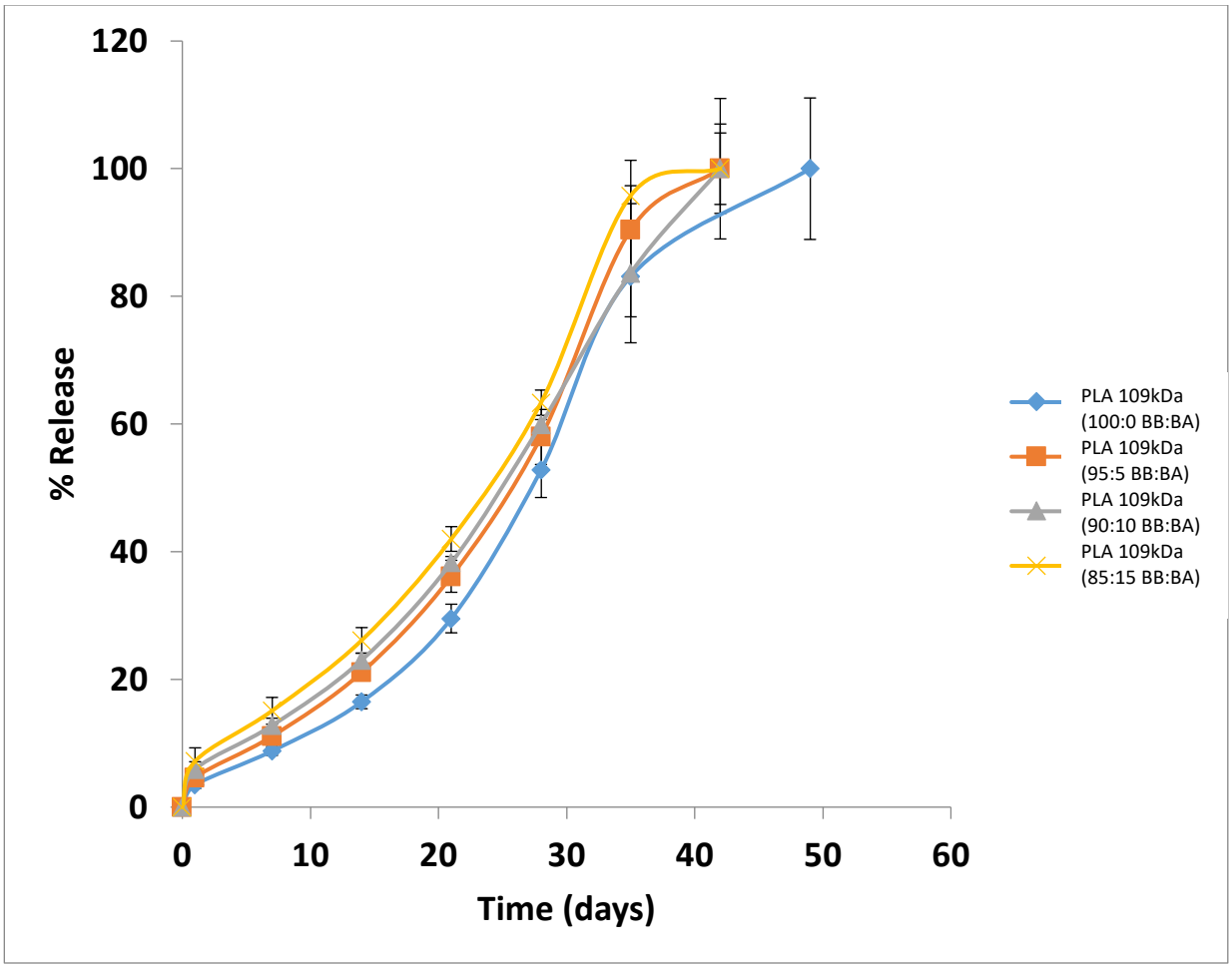

Figure 20. *In vitro* Release Profiles of Rivastigmine Base Comparing Solvent Composition for Formulations of PLA (109 kDa) at 5% (w/v) (n=6).

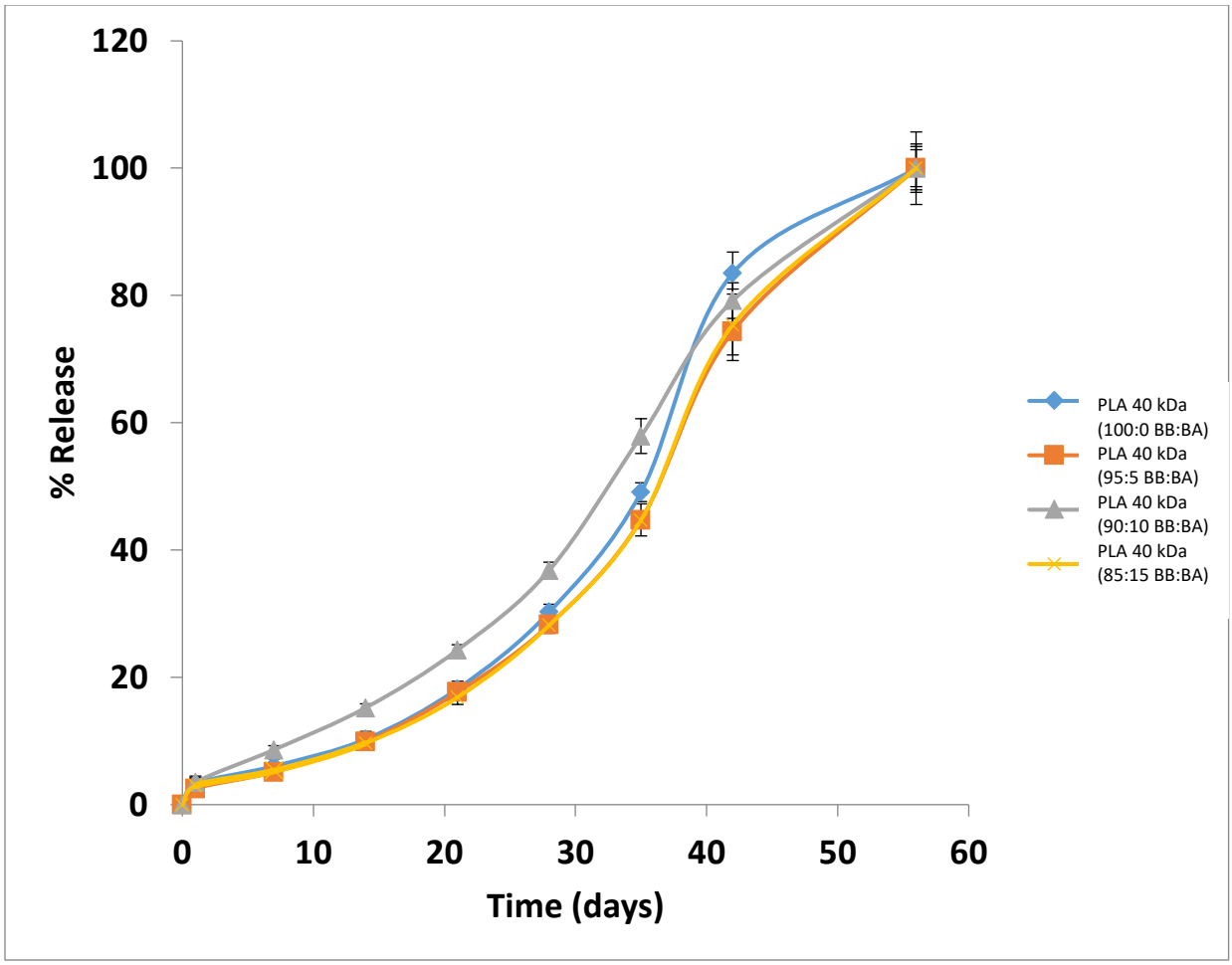

Figure 21. *In vitro* Release Profiles of Rivastigmine Base Comparing Solvent Composition for Formulations of PLA (40 kDa) at 5% (w/v) (n=6).

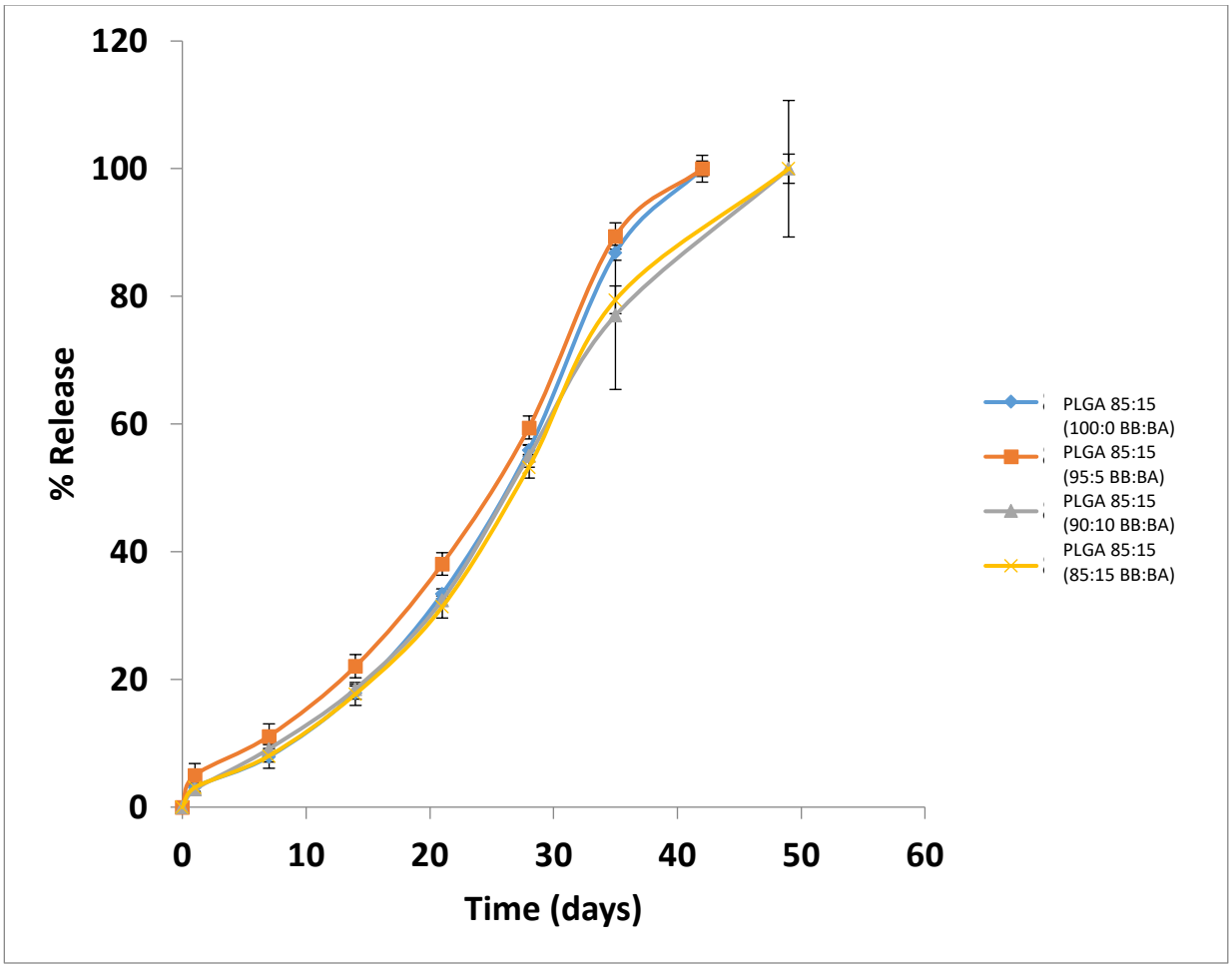

Figure 22. *In vitro* Release Profiles of Rivastigmine Base Comparing Solvent Composition for Formulations of PLGA (85:15) at 5% (w/v) (n=6).

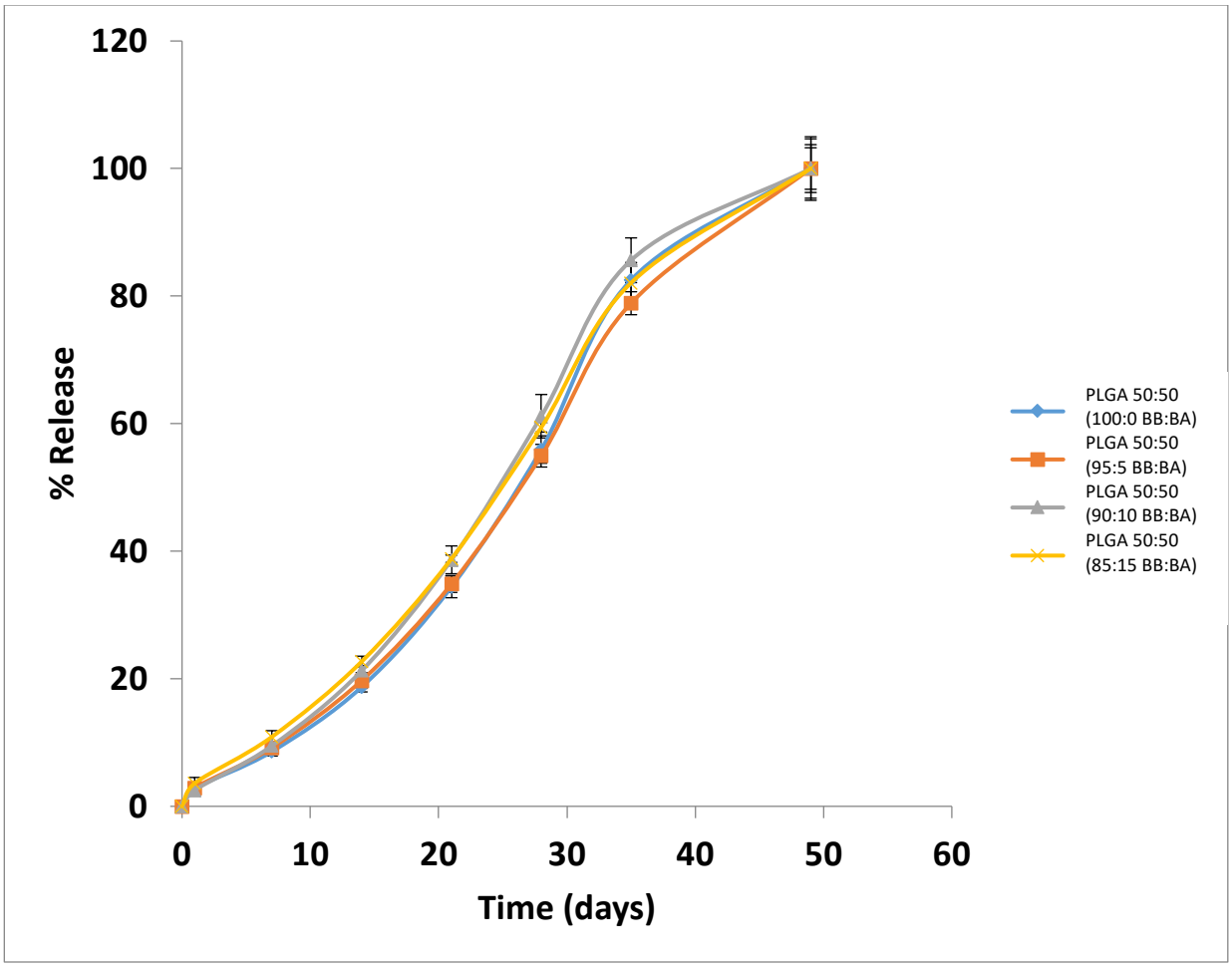

Figure 23. *In vitro* Release Profiles of Rivastigmine Base Comparing Solvent Composition for Formulations of PLGA (50:50) at 5% (w/v) (n=6).

3.16. *In vitro* Release Profile Comparing Therapeutic Hydrophobicity in Phase Sensitive Formulations

Phase sensitive samples were prepared at a PLGA (50:50) polymer concentration of 5% (w/v) in 95:5 benzyl benzoate: benzyl alcohol solvent. Vigorous mixing and sonication allowed for homogenous preparations to be formulated followed with the suspension of rivastigmine tartrate at a concentration of 33.6 mg/ml and 216 mg/ml rivastigmine base. Release was observed over 14 days for rivastigmine tartrate (RT) and 49 days for rivastigmine base (RB), figure 24. Burst release was 26% which is about 8 times higher than that observed for formulations with

hydrophobic rivastigmine base. The amount of rivastigmine tartrate one is able to incorporate into this formulation may be a limiting factor due to its hydrophilicity and the nature of having to suspend the drug molecule in the formulation rather than readily dissolving it within the formulation. However, this rivastigmine tartrate concentration is still enough for 14 days of adequate drug level to be maintained for therapeutic effectiveness at ~2.4 mg/day which will avoid first pass metabolism and act as the equivalent of approximately 4 mg/day dose. At this release rate, the therapeutic window of 1-400 ng/ml can be maintained.

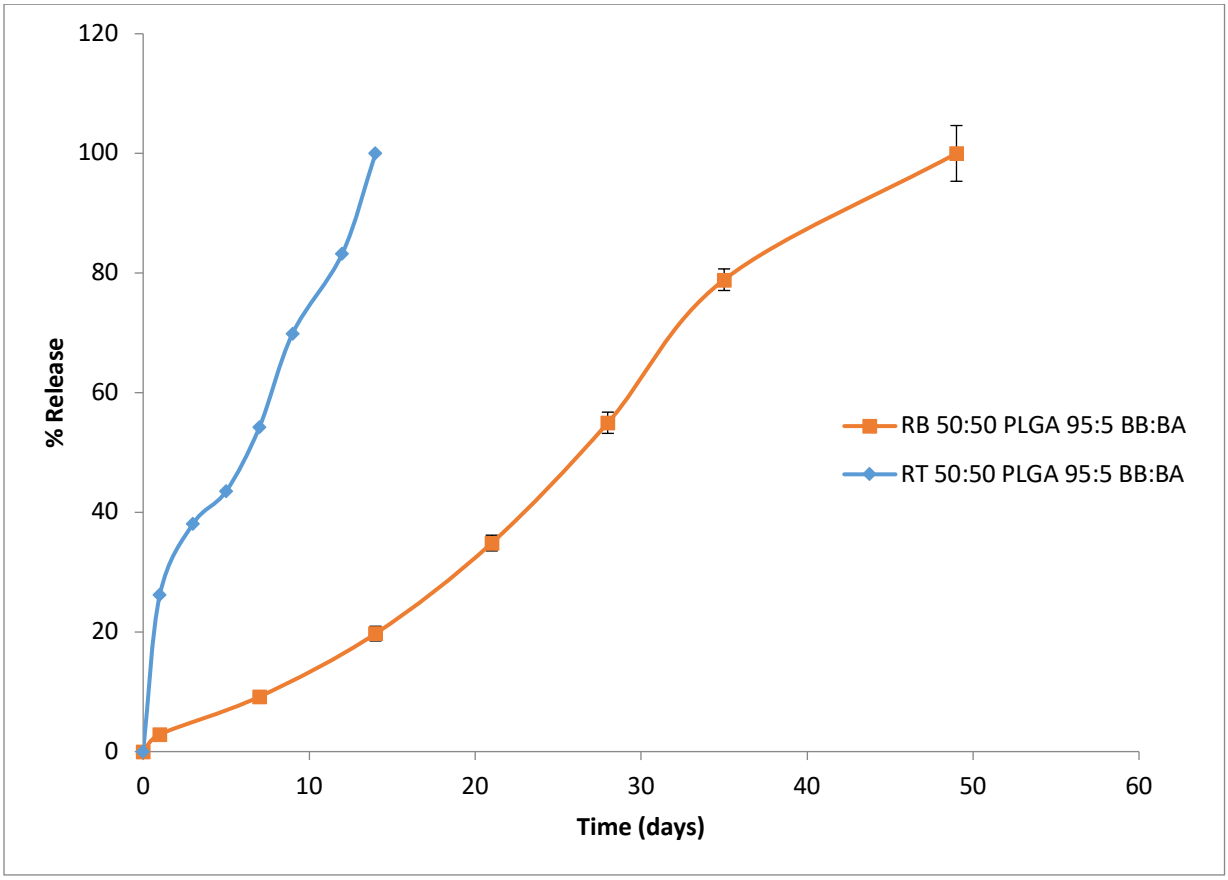

Figure 24. *In vitro* Release Profiles of Rivastigmine Tartrate and Rivastigmine Base Comparing Therapeutic Hydrophobicity (n=6).

3.17. *In vivo* Release Profile of Rivastigmine Tartrate from Phase Sensitive Formulation

While *in vitro* rivastigmine base showed an optimal release, *in vivo* that wasn't the case. We found that *in vivo* the phase sensitive formulation was not able to control the release of

rivastigmine base and instead, as the organic solvent was displaced, so was the hydrophobic drug. Therefore, the rivastigmine tartrate formulation was used for *in vivo* testing. Blood samples were collected from the tail vein of rats at predetermined time points as listed in Table 4. Extraction of rivastigmine tartrate from blood samples was accomplished using acetonitrile and centrifugation. Extracted rivastigmine tartrate was then quantified using RP-HPLC. Release of rivastigmine tartrate was observed through day 7 for the phase sensitive formulation, figure 26. Burst release reached a circulating drug amount of 0.5 mg at time point 15 minutes, figure 25. Release of rivastigmine tartrate from solution was observed to peak quickly to a circulating amount of 0.3 mg, figure 27.

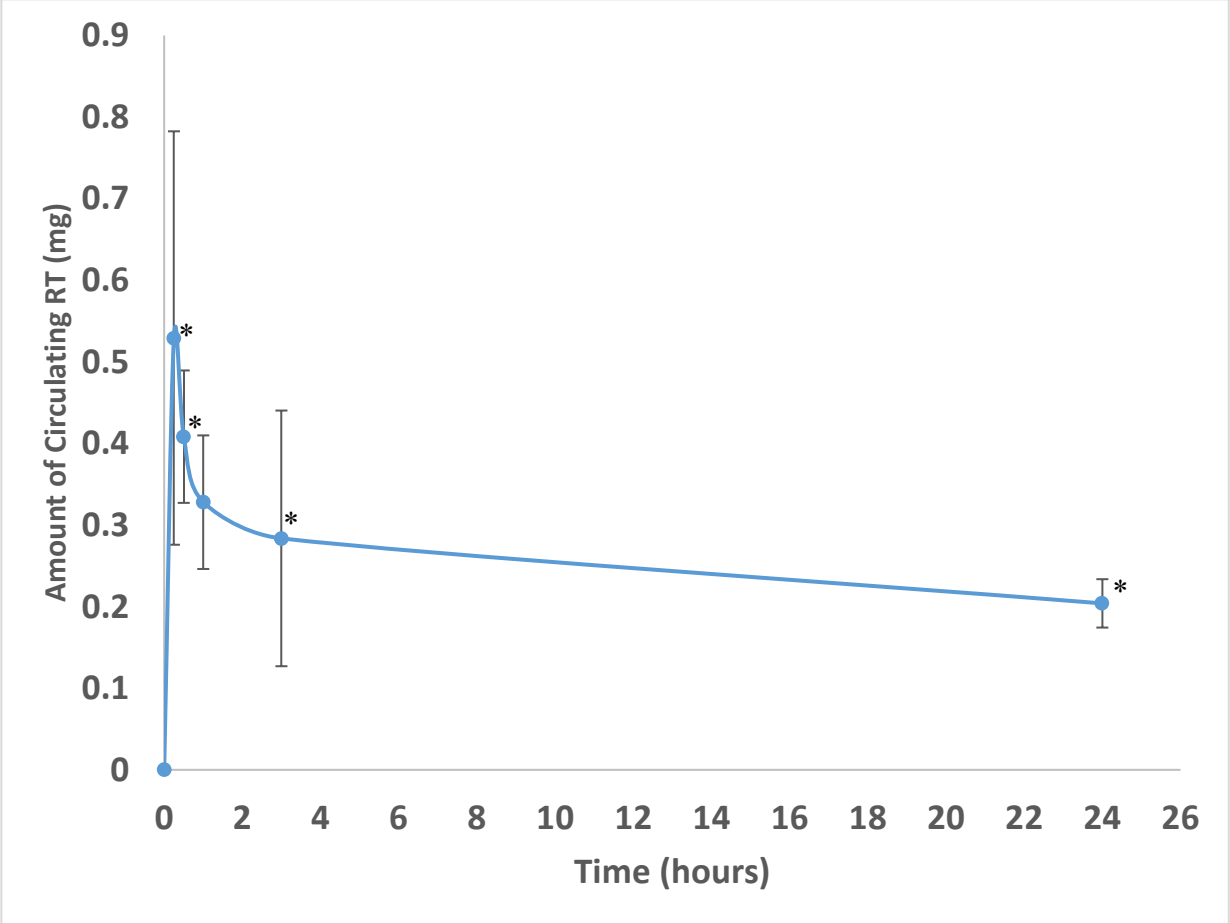

Figure 25. *In vivo* Burst Release of Rivastigmine Tartrate Over the First 24 hrs from Phase Sensitive Formulation (n=6). * indicates a significant difference from formulation group ($p \leq .05$).

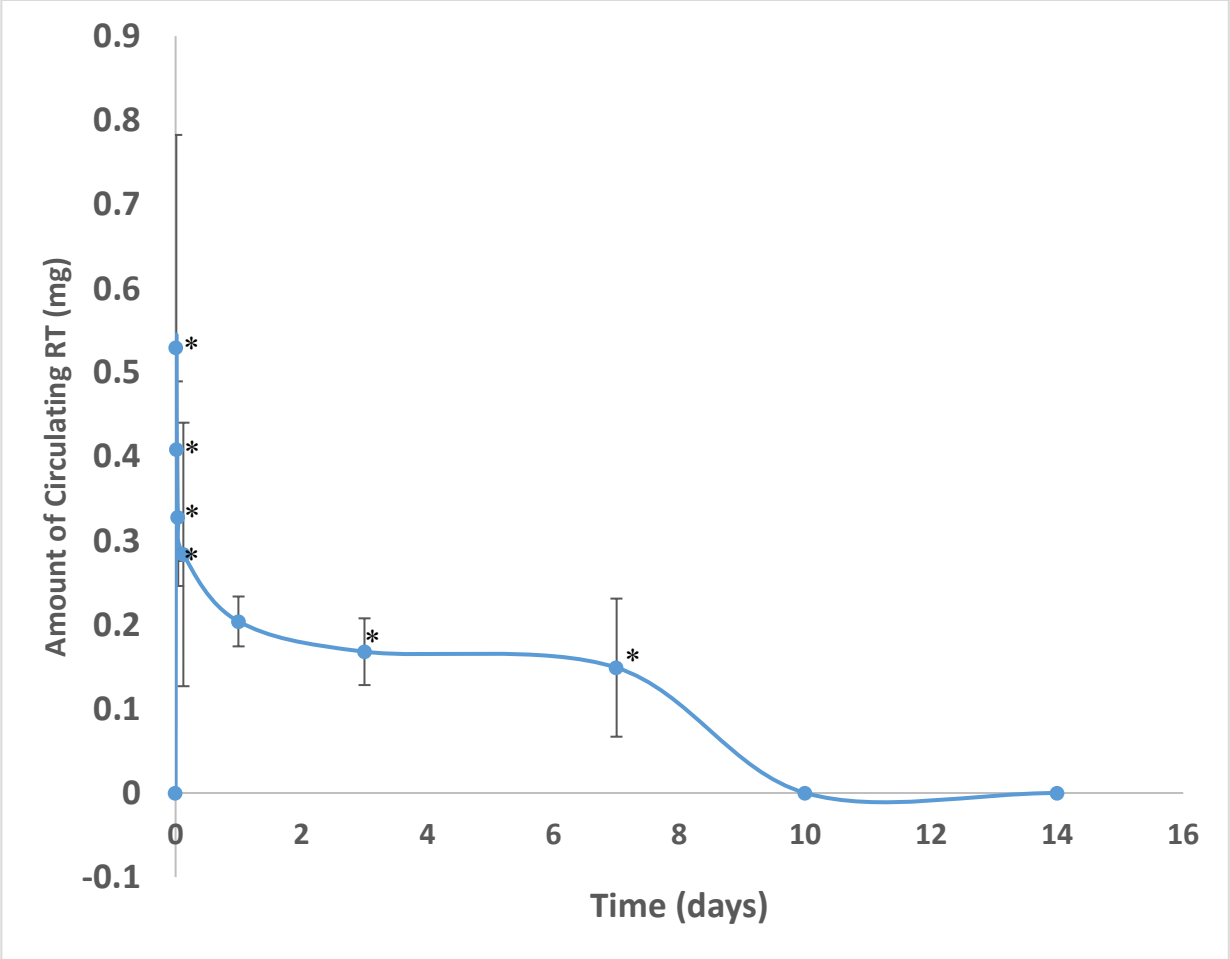

Figure 26. *In vivo* Release Profile of Rivastigmine Tartrate from Phase Sensitive Formulation (n=6). * indicates a significant difference from solution group ($p \leq .05$).

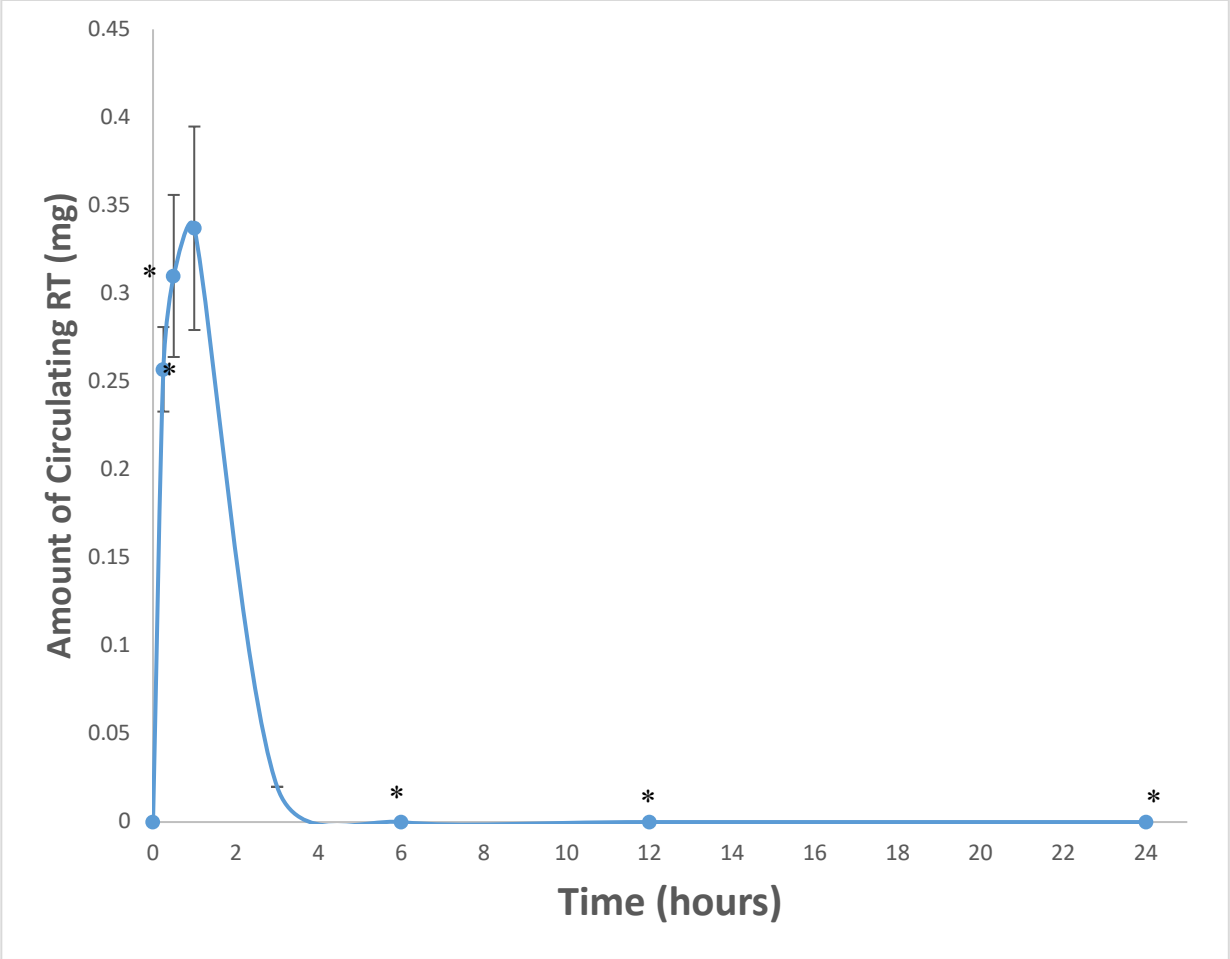

Figure 27. *In vivo* Release Profile of Rivastigmine Tartrate from Solution (n=6). * indicates a significant difference from formulation group ($p \leq .05$).

3.18. Evaluation of Acetylcholinesterase Inhibition

Acetylcholinesterase inhibition was evaluated using Ellman's assay which works on the premise of quantifying products of acetylcholine hydrolysis by acetylcholinesterase. Specifically, Ellman's assay measures the amount of thiol compounds present in a solution. Inhibition of acetylcholinesterase will result in lower quantities of acetylcholine breakdown products which have the thiol moiety that is detected using Ellman's assay. In order to account for potential differences in homogenate samples, micro BCA was employed to normalize all samples. We found that the average thiocholine concentration for healthy control animals was 339 mM. In comparison, the average amount of thiocholine for the solution group was 406 mM 1-day post

administration. The increase of thiocholine for the solution group is likely due to an over compensation of acetylcholinesterase expression and activity in response to the temporary inhibition.[117] The animal groups administered with phase sensitive formulations were sacrificed at 15 minutes, day 3, day 7, and day 14 had thiocholine concentrations of 242 mM, 208 mM, 195 mM, and 265 mM respectively. These results are summarized in table 7.

Table 7. Acetylcholinesterase Activity Upon Administration of Different Formulations.

Sample Group	Time of Sacrifice	Average Concentration of Thiocholine (mM)	Percent AChE Activity
Healthy Control	Day 14	339 ± 1	100%
RT Solution	Day 1	406 ± 3	120%*
Phase Sensitive Formulation	15 minutes	242 ± 15	71%*
Phase Sensitive Formulation	Day 3	208 ± 23	61%*
Phase Sensitive Formulation	Day 7	195 ± 12	58%*
Phase Sensitive Formulation	Day 14	265 ± 17	78%*

Note: Sample number per group was 6. * indicates a significant difference from Healthy Control ($p \leq .05$).

3.19. *In vivo* Biocompatibility

Upon sacrificing animals, the injection site was extracted via dissection and examined visually. Signs of inflammation such as redness, swelling, and vasodilation are evidence of non-biocompatibility. The initial attempt to use the phase sensitive formulation containing rivastigmine base shows massive vasodilation, which when considered with the overdose symptoms observed prior to sacrifice, indicates the formulation is not suitable for further study as it is unable to effectively control release. Phase sensitive formulation with rivastigmine tartrate was used to carry out the study. Slight redness and vasodilation were noticed at the injection site of rivastigmine tartrate solution and phase sensitive formulation groups on day 1 and after 15 minutes, respectively. However, by day 3 and 7 the redness was mostly resolved, and only slight vasodilation was noticeable for the phase sensitive formulation. At day 14 for

phase sensitive formulation and healthy control groups, the signs of inflammation were completely resolved indicating an overall biocompatibility of formulations, figure 28.

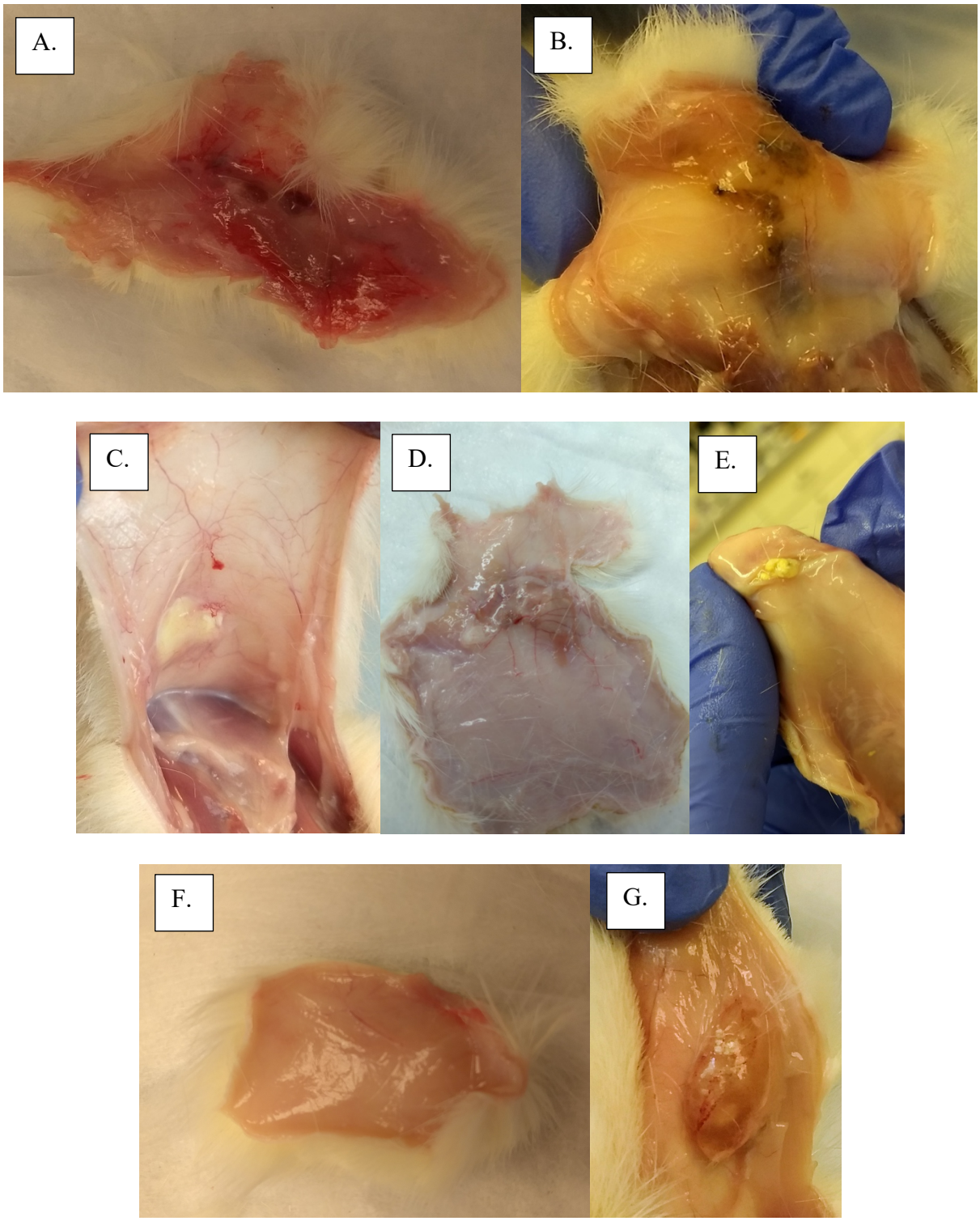

Figure 28. Visual Inspection of Subcutaneous Injection Site for Biocompatibility Determination. Note: Injection site post administration of phase sensitive formulation containing A.) rivastigmine base at 15 minutes. B.) rivastigmine tartrate at 15 minutes. C.) rivastigmine tartrate at day 3. D.) rivastigmine tartrate at day 7. E.) rivastigmine tartrate at day 14. F.) healthy PBS control at day 14. and G.) rivastigmine solution at day 1.

4. DISCUSSION

Controlled release of proteins and peptides in a structurally stable form has been the focus of several investigations over the past decades. Treatment of osteoporosis using controlled release of anti-resorptive peptide sCT has been proposed in this study using thermosensitive, triblock copolymer-based delivery system. Thermosensitive triblock copolymer mPEG-PLGA-mPEG was synthesized using ring opening polymerization followed by diblock condensation. Lactide to glycolide ratios were varied in the PLGA block (3.5:1, 4.5:1, and 5:1) to optimize hydrophobic and hydrophilic characteristics of the copolymer. mPEG is hydrophilic and the molecular weight can be varied to increase or decrease the hydrophilic nature of the copolymer.[92] The PLGA block is hydrophobic with lactide being more hydrophobic than glycolide.[94] Therefore, increasing the block size and/or lactide to glycolide ratio can influence the amphiphilic properties of the copolymer. Furthermore, breakdown of the polymer is attributed to hydrolysis of the PLGA bonds and mPEG to form lactic acid, glycolic acid, and smaller fragments of mPEG.[51,160,161] Breakdown of the copolymer can be slowed by increasing the ratio of lactide to glycolide composition of the PLGA block.[162,163] It has been reported earlier that release from such delivery systems is dependent on diffusion of the incorporated therapeutic and slow controlled breakdown of the copolymer.[38] Consequently, by increasing the ratio of lactide within the PLGA block, the hydrophobicity of the copolymer is increased, allowing decreased rate of copolymer breakdown which extends the release of the incorporated therapeutic. In addition, the weight to volume ratio at which the copolymer is mixed with water can also have an impact on polymer degradation and diffusion of therapeutics, and therefore the release of incorporated therapeutics. Since hydrolysis of the copolymer backbone is key to polymer breakdown, having a higher copolymer content can avoid unnecessary or unwanted hydrolysis.

However, with increasing copolymer content, the copolymeric solution becomes viscous, and consequently the impact on solution injectability needs to be taken into consideration.

Singh et. al. have previously explored variations of thermosensitive triblock copolymers including PLGA-PEG-PLGA and mPEG-PLGA-mPEG with lactide to glycolide ratios up to 3:1 for controlled release of model peptide based therapeutics such as lysozyme and sCT.[14,17,109,110] These previous studies were the basis for exploring lactide to glycolide ratios of 3.5:1, 4.5:1, and 5:1. Initial studies of mPEG-PLGA-mPEG consisted of eleven variations of mPEG-PLGA-mPEG synthesized with serially increasing length of mPEG and PLGA blocks with lactide to glycolide ratios up to 3:1, in order to find a copolymer with the longest hydrophobic PLGA block, while maintaining the desired properties of minimal burst release, controlled release, and complete release of conformationally stable therapeutic.[14] Eleven copolymers were synthesized out of which only four were able to transition from solution to gel form at body temperature, as tested using the test tube inversion method. These were further tested for controlled release and biocompatibility. The release of lysozyme showed the importance of block length in a number of ways.[19] First, it showed how copolymers with smaller mPEG length were able to form more stable gels with lower burst release as well as volume contraction upon expulsion of aqueous phase and push out effect. Second, the larger PLGA block gave insight into its role in slowing degradation of the copolymer which in turn slows release of therapeutic. Larger PLGA block makes the gel more hydrophobic making breakdown, which is primarily due to hydrolysis, more difficult. From this initial study, further testing using sCT was explored in this research using the insight gained.[53] Both lysozyme and sCT retained bioactivity demonstrating the ability of the copolymer to protect the structure of sensitive protein and peptide-based therapeutics. Release of therapeutics was observed over the course of 28 and 42 days for lysozyme and sCT, respectively.

Burst release was minimized to ~22% for lysozyme and ~6% for sCT. The complete details and further insight into the rationale of the current work can be found in those previous publication. [14,17,109,110]

The balance between hydrophobic and hydrophilic blocks in the copolymer is the driving force behind its transition from solution to gel. [14,17,109,110] This delicate balance can be manipulated based on the structural composition of the copolymer. Hydrophobic effect in the presence of increased temperature drives the rearrangement of the hydrophobic and hydrophilic blocks in order to decrease entropy and be energetically favorable. The effects of altering the hydrophobicity of the polymer as a whole are evidenced by the sol-gel transition temperatures observed for each copolymer. In this study, sol to gel transition temperatures of mPEG-PLGA-mPEG copolymers of three different lactide to glycolide ratios were tested using tube inversion method and copolymer with LA:GA 5:1 where phase transition temperature <37 ˚C was found to be appropriate for further development into a controlled release formulation. ^{1}H-NMR spectra confirmed successful synthesis of mPEG-PLGA-mPEG copolymer with lactide to glycolide ratio 5:1. Furthermore, GPC verified fairly uniform distribution of the purified copolymers evident by narrow and symmetrical distribution of retention peak and PDI relatively close to a value of 1. In general, PDI value of less than 2 is considered an optimal polymerization method.[41,19,109,163]

Copolymer mPEG-PLGA-mPEG forms micelles in aqueous solution owing to their amphiphilic nature. Ability of copolymer mPEG-PLGA-mPEG to form micelles is yet another way in which the hydrophobic effect is evident. Amphipathic copolymer chains will rearrange in order to minimize interactions of hydrophobic blocks with the aqueous solvent. The hydrophobic domain induces assembly of the hydrophobic PLGA chains towards the core of the micellar structure and hydrophilic PEG chains facing the aqueous solvent. CMC is an unique

characteristic concentration at which induction of micellar assembly takes place.[157] CMC of mPEG-PL_5GA_1-mPEG (LA:GA, 5:1) thermosensitive triblock copolymer was determined using a fluorimeter with pyrene as the hydrophobic fluorescence probe. Fluorescence of pyrene at increasing copolymer concentration was measured and intensity ratios of peaks at 379 and 393 nm were calculated. Once CMC was reached, a drastic decline in graph was seen due to decreased fluorescence detection of pyrene owing to its entrapment within the micelles. Pyrene is attracted to the hydrophobic environment within the micelles and at CMC micellar assembly allows entrapment of hydrophobic pyrene which can be seen as a sharp decline in intensity ratios of its first peak to the third.[157] The point of sharp decline in fluorescence intensity in figure 4 shows that the CMC of mPEG-PL_5GA_1-mPEG (LA:GA, 5:1) thermosensitive triblock copolymer is 25 μg/mL.

Initial research into thermosensitive delivery systems was limited due to toxicity caused by the use of organic solvents, such as with organogels,[53] and cytotoxicity of the polymers, such as poly(N-isopropyl acryl amide) and poloxamers (polyethylene oxide, polypropylene oxide) due to their inability to biodegrade.[41] The development of mPEG-PLGA-mPEG triblock copolymers greatly improved thermosensitive polymer applicability given their excellent biocompatibility and biodegradation.[17,41,110] Triblock copolymer mPEG-PL_5GA_1-mPEG (LA:GA, 5:1) used in this study showed relative cell viability >80% for up to 1 mg/mL concentration with an IC_{50} >10 mg/ml in HEK 293 cells. The products of polymer breakdown are lactic acid, glycolic acid, and smaller fragments of mPEG which are naturally metabolized and excreted by the body and are therefore highly biocompatible. Furthermore, aqueous solubility of mPEG-PLGA-mPEG avoids the use of toxic organic solvents in the delivery system.

It is not uncommon for controlled release systems to exhibit burst release at or above 20% within the first 24 h.[18] This has primarily been attributed to the amount of drug that lies near the surface of the gel implant and is readily released.[17,19,41] However, when the concentration of polymer and/or the hydrophobic block is altered to increase the overall hydrophobic nature of the polymer, diffusion can be reduced, and the burst release can be minimized. Eventually, breakdown of the polymer allows therapeutic molecules to be released which consequently creates channels to be formed in the gel matrix allowing for subsequent breakdown and diffusion to occur.[29,41] In such instances, the release profile may show a biphasic release or a drastically increased release rate towards the end of release. Release profiles of sCT demonstrated by copolymer mPEG-PL_5GA_1-mPEG (LA:GA, 5:1), used in this study at 30 and 40% (w/v), maintains steady release over the entire duration justifying superior control of the copolymer over release of therapeutic on increasing the hydrophobic to hydrophilic block ratio when compared to other systems such as our previous work using only ~3:1 lactide to glycolide ratio copolymer.[109] In addition, the increased w/v of copolymer in our formulation from 30% to 40% also helps decrease burst release and provide for a longer duration of release. The constant supply of therapeutic will help maintain constant therapeutic level of sCT, thereby avoiding peaks and troughs concomitant to multiple administrations.

In recent years several proteins and peptides have surfaced as an essential category of therapeutic drugs. However, their unique physiochemical and biological properties make them susceptible to chemical and physical degradation. Stability of protein therapeutics is one of the major challenges associated with controlled delivery of such drugs over a prolonged duration. Several enzymes and environmental factors pose challenges *in vivo* necessitating frequent dosing of protein and peptide-based drugs. Thermosensitive copolymer depot based controlled drug

delivery systems overcome stability challenges of protein-based therapeutics alongside providing a controlled release. These copolymers protect the native conformation of the sCT protein structure by masking it from the effect of surrounding environment in depot form. Hence, the protein is maintained and released in its active conformation from such copolymeric depot-based delivery systems. CD spectroscopy confirmed the conformational stability of sCT released from the delivery systems in comparison to freshly prepared sCT solution. Storage stability of sCT incorporated in thermosensitive copolymer stored at 4 ˚C also revealed that sCT maintains its conformational stability in comparison to freshly prepared sCT solution owing to the protective effect of the copolymer incorporating sCT. The results indicate sCT is released from mPEG-PL_5GA_1-mPEG (LA:GA, 5:1) copolymeric depot in its native conformation which is essential for its biological activity.

There are many factors to consider when optimizing a thermosensitive copolymer for controlled delivery of therapeutic. In addition to the parameters considered here, things such as drug hydrophobicity, size, etc. can have major impacts on release profile. Furthermore, some of the parameters that can be changed in an effort to optimize the release system can have impacts that are not directly proportional to the changes studied. Also, there may be points that until reached, no effects are seen and upon reaching, drastic changes can be observed. So, while general knowledge of hydrophobic effect and other physico-chemical characteristics of the drug can give insights to the potential effects of altering the release system, the only way to know for sure how drastic the nature of the polymer and release profile change is will be upon testing.

In a study of thermosensitive PLA-PEG-PLA triblock copolymer by our lab, we further compared effects of drug molecules on degradation behavior and release profile.[63] Hydrophobicity, solubility, and size were compared and contrasted among three model

therapeutics: bovine serum albumin (BSA), insulin, and risperidone. These therapeutics were chosen for their contrasting characteristics where BSA was the largest at ~66,400 Da, insulin was the middle size at 5,808 Da, and risperidone was the smallest at 410 Da. In addition to size, these molecules vary in solubility and hydrophobicity with BSA having a partition coefficient of 0.007 which makes it the most hydrophilic in relation to the other two molecules, insulin having a partition coefficient of 0.02 which again puts it in the middle of the other two drugs in terms of hydrophobicity, and risperidone having a partition coefficient of 3.49 which represents the most hydrophobic of the three molecules. The effects of these variables can be observed in the size of pores that form upon initial and continued release of the drug molecules and the degradation rate of the polymer to influence release. The larger the drug molecule, the larger the pores formed as evidenced by the largest molecule, BSA, having the largest pores and the smallest molecule, risperidone, having the smallest pores. The partition coefficient and hydrophilic nature of BSA further drives the release and generation of larger pores in comparison to the other drug molecules. In addition, the large pores and diffusion driven release of BSA due to its hydrophilicity influence polymer degradation by exposing more polymer surface area to the aqueous environment and propagating hydrolysis. In turn, the consequence is a faster release of BSA in comparison to the other molecules. Risperidone contrasts BSA nicely in how the effects of size and solubility influence release and polymer degradation. With its high hydrophobicity, risperidone does not experience the drive to diffuse into the aqueous environment and this, in addition to its small size leads to less and smaller pores to form. Additionally, it aids in repelling water and slowing the degradation of polymer to further slow the release rate. Insulin is found to fall between the two extremes of BSA and risperidone, as expected.[63] These findings further

support the information mentioned previously and put an extra emphasis on consideration of these factors when designing release systems.[164]

Phase sensitive smart polymers are a great option for controlled delivery of therapeutics. The ease of manipulation to optimize a formulation to suit the desired release kinetics is extremely valuable. The ease of therapeutic incorporation is also a major benefit. Looking at factors such as polymer concentration, drug concentration, polymer composition, solvent composition, and hydrophobicity of the drug molecule can guide the development of the best suited formulation for the drug at hand. Furthermore, testing these variables can uncover characteristics that might otherwise go unnoticed or against what one may expect.

Polymer concentration can be easily varied to explore drug release from a phase sensitive release system. By increasing the weight to volume ratio of polymer to solvent, observations were made from the corresponding release profiles. In terms of hydrophobic effect, the driving force behind everything is entropy. Arranging in a more energetically favorable orientation will allow for a reduction in entropy. However, in the case of polymers, the orientation each monomer is driven to take is typically that of micelles. The micelles usually have an optimal organization in which free energy can be reduced as much as possible. Following the formation of micelles, the micelles themselves can then arrange among each other to again reduce free energy. The problem comes into play when there isn't the correct balance of polymer needed to form optimal micelle structure and subsequent organization. That leads to the results we observe where the balance of polymer for optimal reduction in free energy is met and disturbed. When the correct balance is achieved, and organization is optimal, the release profile reflects this in the improved controlled release of drug molecule which can be attributed to a couple of effects that rely on this organization structure such as ability to diffuse and achievable hydrolysis rate. The

reasoning to support this phenomenon as the cause of release profile variations observed in this study of polymer concentration has been explored extensively throughout discovery of hydrophobic effect and is well demonstrated in the work of Samir Mitragotri and his team.[165]

Drug concentration can influence release profile and be used as a means to obtain a desired release rate over a desired length of time. As seen with the studies performed, increasing drug concentration does not proportionately increase duration of drug release. However, if there is a desired amount of drug release per unit of time, increasing or decreasing drug concentration may be able to influence the result. Other modification to release profile could also be seen when adjusting drug concentration which puts emphasis on examining this factor when developing a delivery system for a certain therapeutic.

As with thermosensitive smart polymers, polymer composition can play a major role on the release profile of a delivery system. We examined four polymer compositions: PLA (109 kDa), PLA (40 kDa), PLGA (85:15), and PLGA (50:50). The basis for this selection was hydrophobicity of each polymer. In the case of PLA with molecular weight of 109 kDa versus PLA with molecular weight of 40 kDa, the increase in molecular weight is going to increase hydrophobic effect. Similarly, for the comparison of PLGA with lactide to glycolide ratio of 85:15 versus PLGA with lactide to glycolide ratio of 50:50, an increase in lactide content will increase hydrophobic effect due to lactide being more hydrophobic than glycolide. In addition to comparisons that can be made between the two PLA polymers and PLGA polymers, comparisons can be made between PLA and PLGA. Addition of glycolide to polymer composition will decrease hydrophobicity which in turn can influence release profile. In all comparisons the increase in hydrophobicity play a major role in decreasing the rate of polymer degradation by repelling water that is responsible for hydrolysis of the polymer. The decreased

rate of hydrolysis will slow the release of drug molecules and result in the observed release profiles where the most hydrophobic polymer, PLA (109 kDa), has the longest duration of release. However, the decrease in hydrolysis can also negatively impact release profile. The biphasic nature of PLA release profiles demonstrates the negative impacts that can occur when initial release is due to diffusion and subsequent release is more dependent on polymer degradation. In the case of PLGA copolymers, the addition of the more hydrophilic component glycolide allows for a balance between diffusion driven release and polymer degradation driven release. This observation adds additional importance when considering polymer composition and the effect on release profile.

Solvent composition is unique to phase sensitive smart polymers and very influential on release profile since the formation of polymer depot is impacted by the hydrophobicity of the solvent and the nature in which it is displaced from the polymer solution. As is expected, the higher hydrophobicity of the solvent will produce a polymer depot that took longer to undergo phase transition. This has many impacts on release profile by influencing the formation of depot and the corresponding physical characteristics such as smoothness of polymer surface and formation of channels in addition to channel size. The physical characteristic will have a direct effect on hydrolysis of polymer and influence polymer degradation driven release. We observed this in our study comparing benzyl benzoate and benzyl alcohol ratios comprising our solvent. In all polymers compared, the increase of benzyl alcohol leads to the increase in burst release and subsequently shorter duration of release in most cases. This result is supported by the fact that benzyl alcohol is more hydrophilic when compared to benzyl benzoate. The increased hydrophilicity of the solvent drives the faster phase transition for depot formation as solvent is displaced into the aqueous environment. The impacts of this faster phase transition are those of

which were mentioned previously. As with polymer concentration, solvent composition can influence release in ways that may not be expected like in the case of PLGA (50:50) at 5% (w/v) where solvent composition of 95:5 BB:BA is more favorable for release profile than that of 100:0 BB:BA as may be expected. This again demonstrates the delicate balance hydrophobic effect has on release profile.

Hydrophobic effect plays the leading role in release profiles of phase sensitive smart polymers. So, it is of no surprise that altering the hydrophobic nature of the drug being studied can have an influence on the release profile observed. Rivastigmine base is hydrophobic while rivastigmine tartrate is hydrophilic. Naturally, incorporating a hydrophobic drug in a hydrophobic release system is easy and can provide controlled release in vitro. However, one must also consider the effects that could be seen once translated *in vivo*. Because the phase transition of these smart polymers relies on the displacement of hydrophobic organic solvent, it is possible that displacement of hydrophobic drug could also take place. Therefore, having a formulation that offers opposing characteristics can be of value when continuing to develop a delivery system. The hydrophilic nature of rivastigmine tartrate conveys the necessity of formulating a suspension rather than a homogenously dissolved solution. Rivastigmine tartrate would not be expected to displace in such a way of rivastigmine base as phase transition occurs but could be driven to diffuse out of the delivery system based on its hydrophilic nature. We observed there is controlled release of rivastigmine tartrate from our phase sensitive delivery system and it contrasts the release profile of rivastigmine base in some ways. For example, rivastigmine base has a lower burst release and a longer duration of release.

The formulation of rivastigmine tartrate in a phase sensitive smart polymer composed of PLGA (50:50) at 5% (w/v) in 95:5 BB:BA was accomplished by suspending the drug in the

smart polymer. Injection volumes of 0.5 ml with a concentration of 4.2 mg/ml were injected subcutaneously at the base of the neck/ shoulder blades of the rats. Rivastigmine base had previously shown promising *in vitro* release, but upon initial testing *in vivo*, control of burst release was not adequate, and the animal had to be sacrificed due to toxicity of high rivastigmine base levels. The inability to minimize burst release is attributed to hydrophobic rivastigmine base displacing with the hydrophobic solvent during phase transition after injection into the aqueous environment in the subcutaneous space. Solution of rivastigmine tartrate was also used as a control in addition to a blank control of PBS for the healthy control group.

Phase sensitive formulation of rivastigmine tartrate provided a continuous, therapeutic level of release over 7 days. Burst release was minimal for the formulation containing rivastigmine tartrate. A total circulating drug amount of 0.5 mg was the maximum drug amount observed during burst release at 15 minutes post administration. The remaining duration of release provided drug amounts in the range of about 0.1 – 0.2 mg. Furthermore, the RT was shown to be bioactive as evidenced by its capability of inhibiting acetylcholinesterase. Healthy controls did not have acetylcholinesterase inhibition since no drug was given at the time of dosing and the acetylcholinesterase activity was determined to be the baseline for healthy rats. Solution group saw an increase in acetylcholinesterase activity at 24 hrs post administration. This may seem counterintuitive, but other research supports these results and suggests that the acute inhibition actually leads to over compensation of acetylcholinesterase activity by increasing expression of this enzyme and its subsequent activity.[117] Furthermore, inhibition of acetylcholinesterase was observed to continue after levels of drug could not be detected up to day 14. This is likely due to a combination of factors such as prolonged exposure to rivastigmine

tartrate and low levels of drug (below the lower limits of detection) continuing to be released as the inner most polymer degrades and hence continuing to exert an inhibitory effect.

In addition, the formulation was shown to be biocompatible as evidenced by visual inspection of the injection site post administration. Inflammation is a side effect typically seen of any injection immediately following dosing. The ability to resolve signs of inflammation, vasodilation, etc. as shown in our study, support the biocompatibility of the formulation. Vasodilation has been well documented as a side effect of benzyl benzoate and it has even been used for just that. Therefore, it is not unexpected to see the vasodilation at the injection site as we do. The vasodilation could have other effects such as increased circulation to further aid displacement of the organic solvent from the polymer depot and increased rate of therapeutic entering the blood system. The vasodilation aided by the presence of benzyl benzoate would also support the unexpected toxicity cause by the rapid release of rivastigmine base from the formulation. The increased circulation would again aid the displacement of the organic solvent, but since rivastigmine base is very hydrophobic, it may partition more into the solvent rather than the polymer depot that is forming. This efflux of solvent and drug would lead to the toxicity of high rivastigmine levels we observed in our initial test of our phase sensitive formulation. Rivastigmine base could also be responsible for increased inflammation observed when compared to rivastigmine tartrate since rivastigmine base is hydrophobic and perhaps more rejected by the immune system when present in a large amount such as that following phase transition. Likewise, it also supports the controlled release and minimal burst release of rivastigmine tartrate. Since rivastigmine tartrate is very hydrophilic, it should partition into the less hydrophobic polymer depot that remains upon phase transition.

5. CONCLUSIONS

Patient compliance for effective and long-term management of chronic diseases such as osteoporosis is a major medical hurdle and patients benefit most when therapeutic levels of drugs are maintained at an optimal concentration in the body without frequent administration. Controlled release delivery systems, particularly subcutaneous depot systems help overcome this hurdle by making the dosing regimen easy, convenient, and consistent. To this end, we have developed and characterized a PEG-PLGA based thermosensitive triblock copolymer for controlled delivery of sCT. The copolymer exhibited exceptional biocompatibility and demonstrated zero-order release profile of sCT over a period of ~60-70 days in a biologically active form. This thermosensitive copolymer-based delivery system could potentially deliver sCT at a controlled rate for up to two months following a single subcutaneous injection, thus improving patient compliance and quality of life in the treatment of osteoporosis. Further studies would entail determination of drug release *in vivo*, efficacy of the formulation in the treatment of osteoporosis in an appropriate animal model as well as long-term biocompatibility. Overall, ease of synthesis and incorporation of therapeutics in the thermosensitive copolymer based controlled delivery system used in this study can potentially change the conventional strategy for delivering various protein and peptide based therapeutic molecules.

Comparisons of copolymer concentration, drug concentration, depot volume, and drug hydrophobicity were made in order to optimize the controlled release of rivastigmine from our thermosensitive smart polymer. From these comparisons, one can see that the optimal formulation in relation to these parameters is going to be that of 35% (w/v) copolymer concentration at a volume of 0.5 ml with a rivastigmine base concentration of 40 mg/ml. With these parameters, a release of about 16 days should be attainable while delivering the appropriate

amount of drug per day. Burst release will be minimized and zero order release will best fit the release profile.

Phase sensitive smart polymers are composed of a biocompatible, organic solvent and biocompatible polymers. Following injection, the biocompatible, organic solvent is displaced, leaving any incorporated therapeutic entrapped and protected within the polymer depot. Its release becomes dependent upon diffusion and breakdown of the polymer over an extended period of time. The ability to adjust the polymer composition as well as the solvent composition allows this smart polymer to be very versatile and easily manipulated to provide the best release profile for the therapeutic of interest. In addition, polymer concentration, drug concentration, and drug hydrophobicity can also be altered to reach the desired release profile.

By examining these factors, we have observed the impacts they have on release profile by altering the hydrophobicity of the release system. From out studies we can draw the conclusion that polymer concentration plays a major role in release profile and should be optimized for optimal influence of hydrophobic effect. We found the best concentration to be 5% (w/v). As for drug concentration, the effects are not proportion to the change of drug concentration. Solvent composition is another major influence on release profile and altering the hydrophobicity by altering solvent composition can allow for further optimization of release profile. In our studies we found an optimal solvent composition of 95:5 BB:BA. Likewise, the polymer composition can also be altered to change the hydrophobicity of the release system as a means of fine-tuning release of incorporated therapeutic. While PLA polymers offer lengthened release, PLGA copolymers may be better suited given they better follow zero order release kinetics. Thought should also be given to the nature of the drug molecule itself. Hydrophilic drug molecules tend to release based on diffusion to the aqueous environment while hydrophobic drug molecules would

be more prone to displacement with the hydrophobic organic solvent and release thereafter based on polymer degradation. Upon analysis of all factors studied, the polymer and solvent concentration and composition combination that may offer the best profile is that of PLGA (50:50) at 5% in 95:5 BB:BA. Hydrophobic rivastigmine base may give longer duration of release, but rivastigmine tartrate also has its merits.

The *in vivo* testing of the phase sensitive formulation for rivastigmine tartrate demonstrates its ability to act as a controlled release delivery system for rivastigmine tartrate. The formulation was able to deliver RT over the course of 7 days at a relatively constant rate to maintain the drug level circulating throughout the body. Burst release was observed to peak at a drug amount of 0.5 mg at 15 minutes post administration. Negative effects were not observed as a result of this burst release which further supports its promise as a delivery system. In addition, the formulation was biocompatible and biodegradable to allow the formulation to be easily resorbed and prevent the need for explanation as is needed for controlled delivery implants.

The current formulations on the market for rivastigmine to treat Alzheimer's disease have a maximum dosing interval of 24 hrs. Our formulation would increase the dosing interval to 7 days and allow for less rise and fall of drug levels to better maintain therapeutic drug levels and symptom relief. The phase sensitive formulation of rivastigmine tartrate is a promising advancement in the treatment of Alzheimer's disease.

6. SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

6.1. Summary and Conclusions

Smart polymers offer many advantages as controlled release delivery systems. Smart polymers are highly optimizable which allow them to accommodate many therapeutics encompassing all characteristics. In addition, smart polymers such as those that are thermosensitive and phase sensitive, can be composed of biocompatible, bioresorbable polymers and solvents including PLA, PGA, PEG, benzyl benzoate, benzyl alcohol, and water. Biocompatible, bioresorbable polymers and solvents allow the delivery systems to be injectable and avoids the need to remove a device or depot as needed in other delivery systems.

Throughout the studies performed, we have demonstrated the ability of thermosensitive triblock copolymers and phase sensitive smart polymers to act as controlled release delivery systems. The thermosensitive copolymer explored was mPEG-PLGA-mPEG (5:1, PLA:PGA). We demonstrated its ability to control the release of salmon calcitonin while protecting it from degradation in order to deliver bioactive therapeutic. The controlled release of salmon calcitonin could be used to treat osteoporosis. In addition, the thermosensitive copolymer was able to control the release of rivastigmine tartrate and rivastigmine base *in vitro*. The main variables examined to optimize the thermosensitive system were the lactide to glycolide ratio, polymer concentration, therapeutic concentration, and therapeutic hydrophobicity. The optimization of the factors allowed us to increase the hydrophobicity of the copolymer in an effort to slow the breakdown and subsequent release of therapeutic to control the burst release and maintain a steady release over an extended period of time. Similarly, phase sensitive smart polymer optimization was examined as a way to deliver rivastigmine following the best release kinetics possible to maintain therapeutic drug levels *in vivo*. Factors such as polymer composition,

polymer concentration, solvent composition, therapeutic hydrophobicity, and therapeutic concentration were used to optimize a phase sensitive formulation to be tested *in vivo*. While a formulation of PLGA (50:50) at 5% (w/v) in 95:5 BB:BA for rivastigmine base had promising *in vitro* results, *in vivo* the formulation was not able to minimize the burst release to a level that is nontoxic. The same formulation with rivastigmine tartrate was therefore employed to overcome the displacement of hydrophobic drug with the hydrophobic solvent upon phase transition. The release profile of rivastigmine tartrate *in vivo* offered a minimal burst release and subsequent controlled release over 7 days. Bioactive rivastigmine tartrate was release from the phase sensitive formulation and evidenced by the inhibition of acetylcholinesterase.

The final conclusions that can be drawn from these studies and support the plethora of other studies conducted by other researchers, is that smart polymers hold promise as controlled release delivery systems. We showed thermosensitive smart polymer potential *in vitro* for delivery of salmon calcitonin for the treatment of osteoporosis. We then showed phase sensitive smart polymer potential *in vitro* and *in vivo* for delivery of rivastigmine for the treatment of Alzheimer's disease.

6.2. Future Directions

The future of smart polymers is promising. Their ability to be optimized allow for tailoring of delivery system for the therapeutic at hand. Further work on using our thermosensitive smart polymer for delivery of salmon calcitonin to treat osteoporosis could be performed in the future to verify our results *in vivo*. In addition, our promising results *in vivo* for the rivastigmine tartrate to treat Alzheimer's disease could be further explored in the future to examine if the duration of release could be extended by using polymers with increasing PLA and PEG chain lengths. Care would need to be taken so burst release doesn't become problematic as

we saw with rivastigmine base possible making prodrug of rivastigmine of larger size in order to restrict diffusion of the drug from the polymer matrix. Further, it is important to explore the drug concentration and enzyme activity in live animal using In vivo microdialysis technique.

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